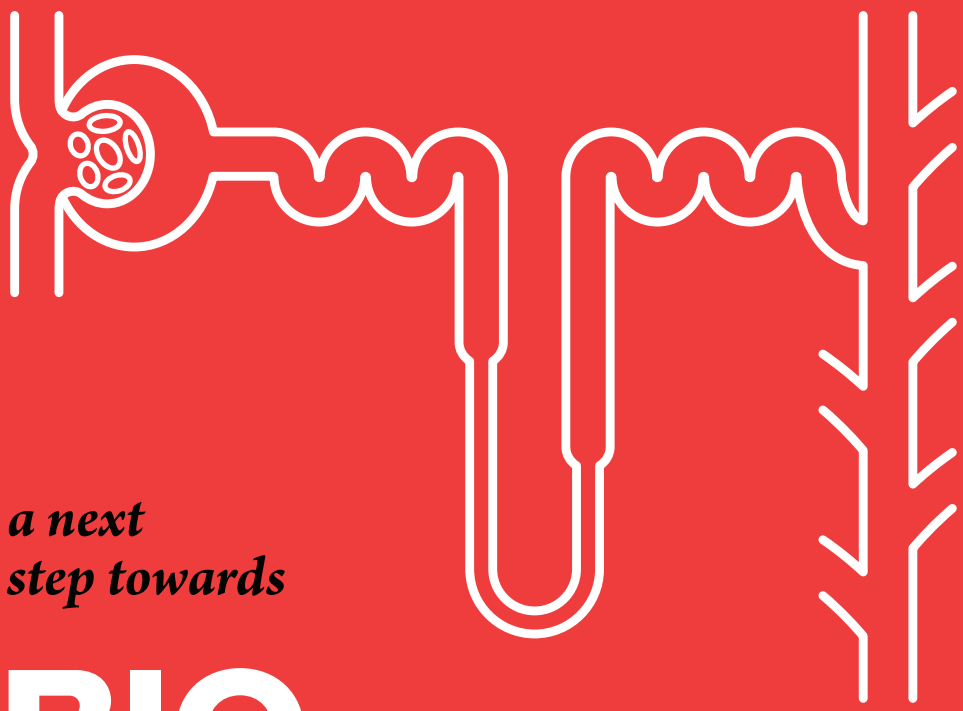

a next step towards

BIO ARTIFICIAL KIDNEY

preclinical safety evaluation

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Layout and cover design: Design Your Thesis, Rotterdam, the Netherlands
Printed by: Ipskamp Printing, Enschede, the Netherlands
ISBN: 978-90-393-7008-7

Financial support by the Utrecht Institute for Pharmaceutical Sciences and the Dutch Kidney Foundation for the printing of this thesis is gratefully acknowledged.

The research presented in this thesis was performed at the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University and the Department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences, Nijmegen.

This research project was financially supported by the Marie Skłodowska-Curie Initial Training Network of the European Union's Seventh Framework Program - BIOART (grant no. 316690), and the Netherlands Organisation for Health Research and Development (ZonMW – 114024113).

A NEXT STEP TOWARDS BIOARTIFICIAL KIDNEY: PRECLINICAL SAFETY EVALUATION

EEN NIEUWE STAP OP WEG NAAR DE BIOLOGISCHE KUNSTNIER: PREKLINISCH VEILIGHEIDSONDERZOEK

(met een samenvatting in het Nederlands)

P R O E F S C H R I F T

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 25 juni 2018 des middags te 4.15 uur

door

Miloš Mihajlović

geboren op 11 april 1989
te Požarevac, Servië

Promotoren: Prof. dr. R. Masereeuw
Prof. dr. L.B. Hilbrands

"Ништа не чини човека толико срећним као његово поштено уверење да је учинио све што је могао улажући у свој рад своје најбоље способности."

-Михајло Пупин-

"Nothing makes a man as happy as his genuine belief that he has done everything he could by investing his best abilities in his own work."

-Mihajlo Pupin-

To my parents.

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CHAPTER 1

GENERAL INTRODUCTION

KIDNEY PHYSIOLOGY

Kidneys are paired retroperitoneal organs and represent an important part of the urinary system. As such, they perform an essential role in filtering blood and producing urine. With the approximate filtration rate of 125 ml/min, kidneys are able to filter around 180 l of blood per day. By reabsorbing most of the filtered water and constituents essential for normal body function, about 1.8 l of urine containing waste products is produced every day. Therefore, it is clear that the kidneys are fundamental for whole body homeostasis. In particular, their main tasks are to regulate acid-base balance, water and electrolyte balance, volume and blood pressure [1-6]. All these functions are enabled by the presence of the well-designed functional units of the kidneys, termed nephrons. On average, each kidney contains 1 million of them. From a structural point of view, nephrons are made of several components including the renal corpuscle, consisting of glomerulus and Bowman's capsule, and the tubular part formed by the proximal tubule, the loop of Henle, the distal convoluted tubule and the collecting duct (Figure 1.1a). The glomerulus, which is formed by a dense network of capillaries, is the main site of the ultrafiltration. The endothelial layer of the glomerular capillaries, together with the glomerular basement membrane and the foot processes of the podocyte form a semipermeable membrane, which under high hydrostatic pressure allows production of the so-called ultrafiltrate, containing small water soluble molecules, but restricts the transfer of cells and large proteins. Further, the ultrafiltrate is subjected to reabsorption of water, electrolytes, glucose and amino acids in the proximal tubule of the nephron [1,7]. In the proximal tubule, smaller sized proteins that have passed through the glomerular filtration barrier are reabsorbed as well, mainly via receptor-mediated endocytosis [8]. Moreover, in the proximal tubule many drugs and other xenobiotics, as well as endogenous metabolic waste products, are actively secreted from the blood and the interstitium in the pro-urine, by multiple influx and efflux transport proteins present in the proximal tubule epithelial cells (PTEC) [9] (Figure 1.1b). The influx of organic anionic solutes is mediated by members of the solute carrier (SLC) family, known as organic anion transporter 1 and 3 (OAT1/*SLC22A6* and OAT3/*SLC22A8*) and the bidirectional organic anion transporting peptide 4C1 (OATP4C1/*SLCO4C1*) located at the basolateral membrane of PTEC [9-13]. The apical efflux of organic anions is enabled by members of the Adenosine triphosphate (ATP)-binding cassette (ABC) transporters including the multidrug resistance proteins 2 and 4 (MRP2/*ABCC2* and MRP4/*ABCC4*) and breast cancer resistance protein (BCRP/*ABCG2*) [14,15]. In addition, the transport of organic anions is facilitated by OAT4 (*SLC22A11*) and the urate reuptake transporter 1 (URAT1/*SLC22A12*) [16,17]. Organic cations are taken up by PTEC by means of the SLC22 family of organic cation transporters (OCT), including OCT1 (*SLC22A1*), OCT2 (*SLC22A2*) and OCT3 (*SLC22A3*), of which OCT2 is in humans the most relevant and highly expressed transporter in the kidney [18-21].The efflux of organic cations is mediated by apical transporters including the multidrug and toxin extrusion proteins 1 and 2 (MATE1/*SLC47A1* and MATE2/*SLC47A2*), organic cation/carnitine transporters 1 and 2 (OCTN1/*SLC22A4* and OCTN2/*SLC22A5*), and the ABC transporters P-glycoprotein (P-gp/*ABCB1*) and BCRP (*ABCG2*) [18,22-26]. Downstream of the proximal tubule, an additional amount of water and ions (Na^+, Cl^-, K^+, Ca_2^+, HCO_3^-)

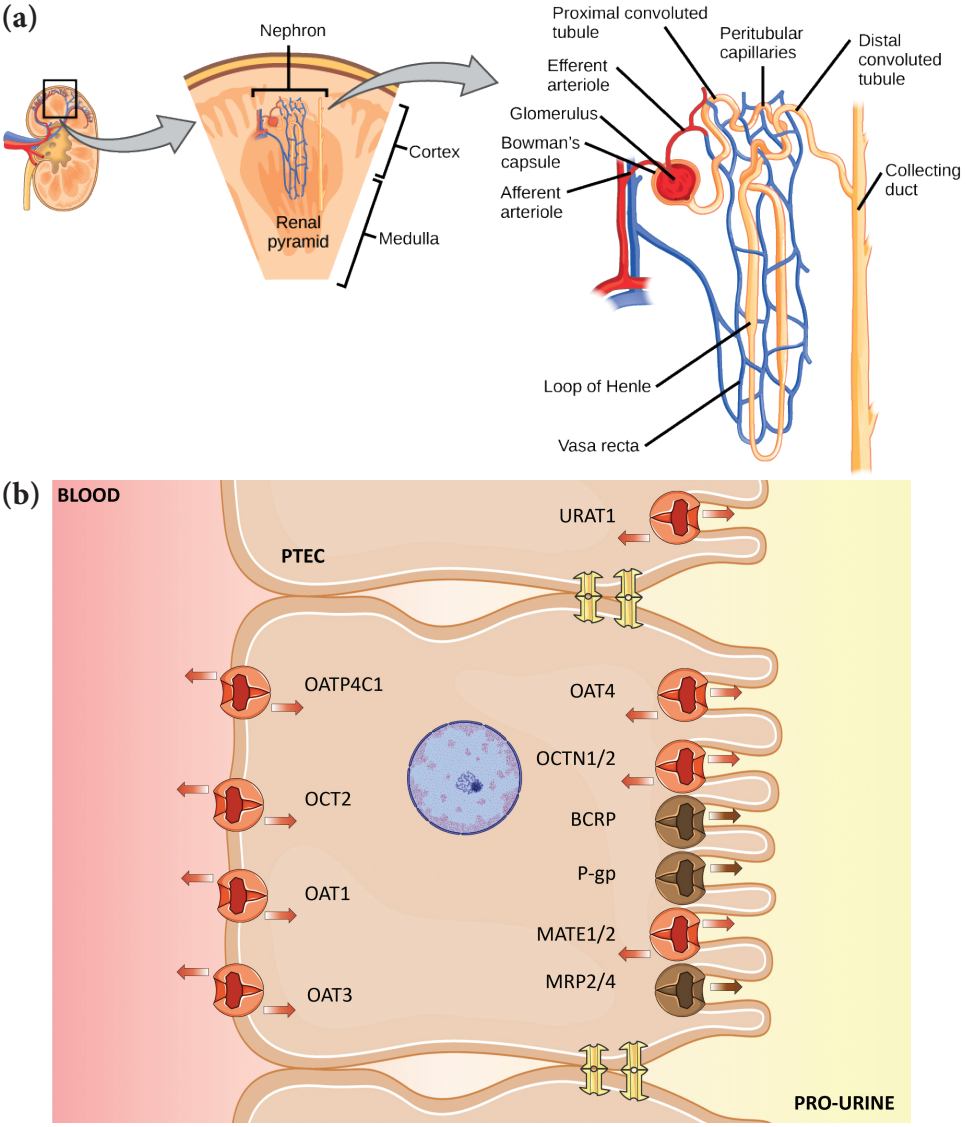

Figure 1.1. Nephron structure and transporter system in human proximal tubule epithelial cells (PTEC). (**a**) Nephrons are divided in five sections: glomerulus, proximal tubule, loop of Henle, distal convoluted tubule, and collecting duct. (**b**) Schematic representation of organic anion and cation transporters in PTEC. Solute carrier (SLC) transporters are depicted in red and ATP-binding cassette (ABC) transporters in brown. Influx transporters expressed at the basolateral membrane: organic anion transporting peptide 4C1 (OATP4C1), organic cation transporter 2 (OCT2) and organic anion transporters 1 and 3 (OAT1/3). Efflux transporters located at the apical membrane: urate reuptake transporter 1 (URAT1), OAT4, organic cations/carnitine transporters 1 and 2 (OCTN1/2), breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), multidrug and toxin extrusion proteins 1 and 2 (MATE1/2) and multidrug resistance proteins 2 and 4 (MRP2/4).

is reabsorbed from the filtrate to the blood within the loop of Henle, the distal convoluted tubule and the collecting duct system, thus concentrating the urine and contributing to the fluid and ion homeostasis. The finally concentrated urine is transported and eliminated via the renal pelvis, ureter and urinary bladder [1-6,9]. In addition to their excretory capacity, kidneys have endocrine functions reflected by the production and secretion of hormones, such as erythropoietin, renin and 1α,25-dihydroxy-vitamin D_3, also known as calcitriol [27-29].

KIDNEY DISEASE

Considering the aforementioned main functions of the kidney, it is clear how kidney diseases can give rise to severe clinical manifestations. There are various types of kidney diseases and causes of kidney damage such as diabetes mellitus, hypertension, inflammatory conditions, including interstitial nephritis and glomerulonephritis, polycystic kidney disease, obstructions of the urinary tract, vesicoureteral reflux, and pyelonephritis [30-36]. With the increasing life expectancy of the general population on the one hand, and the growing rate of various predisposing conditions like obesity and high blood pressure on the other hand, the prevalence of kidney disease is destined to rise. Currently, the prevalence of chronic kidney disease (CKD), defined as a gradual and progressive loss of renal function, is estimated to be between 11 and 13% [37]. One of the main indicators of kidney function is glomerular filtration rate (GFR), defined as the volume of blood filtered by glomerular capillaries per unit of time (ml/min/1.73m^2) [38]. Based on the GFR values there are 5 distinguishable stages of CKD (Table 1.1).

Table 1.1. GFR-based classification [39] and global prevalence [37] of CKD.

CKD stage (description)	GFR (ml/min/1.73m^2)	Prevalence (%)
Stage 1	> 90	3.5
Stage 2 (Mild CKD)	60-89	3.9
Stage 3 (Moderate CKD)	30-59	7.6
Stage 4 (Severe CKD)	15-29	0.4
Stage 5 (End-stage renal disease; ESRD)	< 15	0.1

The clinical picture of various CKD stages is progressing with virtually no symptoms present and no treatment required in stage 1 (GFR > 90 ml/min/1.73m^2), compared to end-stage renal disease (ESRD; stage 5) in which patients present a complex clinical picture and inevitably require some of the available treatment options in order to survive [39]. Some of the most notable complications and co-morbidities of CKD, which to a certain degree can be observed in various stages of the disease, are anemia, mineral and bone disorders, cardiovascular disease, and dyslipidemia [30,31,40]. All of them originate as a direct or indirect consequence of reduced kidney function. Namely, anemia is predominantly caused by a reduced erythropoietin

synthesis, bone and mineral disorders are mostly due to the insufficient vitamin D activation and hyperphosphatemia, cardiovascular problems are due to the alterations in blood pressure and accumulation of uremic waste metabolites in the circulation, while dyslipidemia can be attributed to reduced catabolism of lipoproteins and triglycerides [40-46]. As expected, all of these complications impose a significant health risk and contribute to the high mortality within the CKD and especially the ESRD population [47].

It should be noted that acute kidney injury (AKI), defined as a sudden decline of renal filtration function, is another type of kidney disease which, unlike CKD, is reversible [48]. Major causes of AKI are renal hypoperfusion and exposure to toxic agents such as iodinated contrast media or certain drugs [48-51]. However, even though it is reversible, the mortality due to AKI is not insignificant and the predisposition and risk to develop CKD after restoration of normal kidney function are increased [48,52,53].

CURRENT CKD TREATMENT OPTIONS AND LIMITATIONS

The most complete recovery of function can be obtained by kidney transplantation from either a living or deceased donor. However, there is a significant shortage of kidney donors, resulting in long waiting lists. After transplantation, there can be several causes of more or less rapid decline in kidney function, mainly T cell- and/or antibody-mediated rejection, recurrence of the original kidney disease, ureteric obstructions, drug toxicity, and an infection with the BK virus [54, 55]. To prevent or delay kidney rejection, patients have to use immunosuppressive drugs, but these agents can also increase the risk of other health problems such as infections and malignancies [56,57].

Other alternatives of renal replacement therapy are hemo- and peritoneal dialyses. Hemodialysis relies on the processes of diffusion, ultrafiltration and convection to clean blood from various waste products, to remove excess body fluid, and to correct electrolyte balance, by means of an artificial kidney containing permeable hollow fiber membranes [58]. Typical hemodialysis treatments are performed 3 times a week, with each session lasting 4 h, and this is one of the main drawbacks of hemodialysis as it forms a social and economic burden and reduces patient's quality of life [59,60]. Additional problems of hemodialysis are complications related to arteriovenous fistulae or hemodialysis catheters used to create vascular access, such as clotting, stenosis, and infections [61-63]. In peritoneal dialysis, the peritoneal membrane is used to remove waste products by diffusion and convection into dialysis fluid in the peritoneal cavity. The fluid exchange in this case is performed multiple times per day and can be both manual and automated [64]. Also in this case a main clinical complication is infection, especially peritonitis. In addition to several practical complications of hemodialysis and peritoneal diaysis, these techniques have limited efficacy in removing retention uremic solutes [45]. Specifically, while small water-soluble compounds are easily removed by hemodialysis, middle molecules would require dialyzers with larger pore size membranes for more efficient removal. However, regardless of pore size of membranes, protein-bound toxins cannot be efficiently removed [45]. Moreover, dialysis treatment does not offer any replacement of kidney endocrine functions, including erythropoietin and 1α,25-dihydroxy-vitamin D_3 production [65].

ACCUMULATION OF UREMIC TOXINS

As mentioned earlier, reduced kidney function in CKD, and especially in ESRD, is characterized by incomplete removal of xenobiotics and metabolic waste products, also called uremic toxins, leading to their progressive accumulation [45]. Since many of the toxins have at least some biologic activity, it does not come as a surprise that their accumulation can lead to several complications observed in CKD [66]. Some of those are related to cardiovascular disorders, immune dysregulation, neurologic disorders, protein energy wasting, and even progression of CKD itself [67]. Uremic waste molecules can be classified as small water-soluble compounds with molecular weight below 500 Da (e.g. urea, creatinine, uric acid etc.), protein-bound toxins (e.g. indoles, cresols, hippurates etc.), and middle molecules with molecular weight higher than 500 Da (e.g. β_2-microglobulin, interleukins (IL)-6, 10, 18, 1β, and other small peptides) [66,68]. While small water-soluble compounds are easily removed via standard hemodialyis, the middle molecules require the use of large-pore dialyzer membranes. However, the protein-bound toxins are very difficult to remove because the binding proteins do not pass the pores and only a small fraction of these molecules is present as free fraction (often <10%) [45].

Large part of the uremic toxins are derived from intestinal breakdown of proteins and amino acids by gut microbiota [67,69]. In CKD, there is an imbalanced intestinal microbial community characterized by altered composition and metabolic activity of gut microbiota, also known as dysbiosis, which is mostly due to the use of antibiotics, slow intestinal transit time and/or low consumption of dietary fibers [70-75]. Hence, it is understandable how this can affect the accumulation of uremic toxins in CKD patients. In particular, increased protein consumption could lead to the presence of undigested proteins in the intestine with consequent increased production of various toxin groups, including amines, phenols, indoles, thiols, and ammonia [67]. For example, the prevalence of proteolytic bacteria observed in CKD and ESRD patients, leads to an increased production of the enzyme L-tryptophanase that, starting from L-tryptophan, mediates the formation of indole, which subsequently after reaching the liver via portal venous system is converted by hepatic metabolizing enzymes to indoxyl sulfate [69,76]. Indoxyl sulfate is one of the most studied organic anion uremic toxins and it has been shown to be involved in cardiovascular problems within the CKD population, due to its promoting effects on aortic calcification, vascular stiffness, oxidative stress in endothelial cells and vascular smooth muscle cell proliferation, and decreasing erythropoietin production, thus contributing to the increased cardiovascular mortality [77-81].

Another highly studied toxin which belongs to the group of phenols is p-cresyl sulfate, which also originates from gut microbiota that produce phenolic compounds starting from tyrosine and phenylalanine [82]. Being a biologically active compound and protein-bound toxin, its excessive accumulation in the body over time can lead to various complications. This includes cardiovascular morbidities, renal tubular damage and kidney fibrosis, predominantly caused by oxidative stress in leukocytes, endothelial cells and cardiomyocytes, as well as excessive amounts of reactive oxygen species production and inflammation in renal tubule cells [83-87].

ADVANCES IN BIOARTIFICIAL KIDNEY DEVELOPMENT AND OTHER NOVEL RENAL REPLACEMENT THERAPIES

In light of previously mentioned limitations of currently employed renal replacement therapies it is clear that novel, innovative treatment strategies are desired. A promising example of a new therapeutic modality to treat advanced CKD is given by a bioartificial kidney (BAK). BAK represents a biotechnological and tissue engineering approach aimed at substituting not only the excretory but also the endocrine, metabolic and regulatory functions of the kidney, which are completely absent in the regular dialysis treatment. In order to achieve this effect, BAK relies on the combination of a specific type of polymer membrane, that can be considered as a scaffold, with a cellular component, namely PTEC grown on the polymeric tubular scaffolds to create bioartificial tubules [65]. Considering that dialytic techniques can to some extent only replace glomerular filtration, a BAK device would predominantly replace proximal tubule functions. Therefore, incorporation of a regular hemodialysis filter in a BAK device appears to be the most promising approach.

The first attempts to create a BAK device were made by Aebischer *et al.* who showed that continuous ultrafiltration can be maintained for long periods of time in the absence of anticoagulation, using animal-derived PTEC lines [88]. The next significant contribution to the field of BAK engineering came from Humes *et al.* who successfully cultured dog-derived, Madin-Darby Canine Kidney (MDCK) PTEC as a confluent monolayer inside the lumen of hollow fiber membranes, showing functional transport capabilities and persistent cell viability [89,90]. Next, they managed to create the up-scaled bioartificial renal-assisted tubule device by growing porcine PTEC in polysulfone immunoisolating membranes and showing *in vitro* the transport activities regarding glucose, Na^+, HCO_3^- and organic anions, as well as synthesis of 1α,25-dihydroxy-vitamin D_3 [91]. Following these findings, a study performed in uremic dogs using the standard hemodialysis system in series with a renal assist device containing porcine PTEC proved the efficacy of the device by showing the active transport of water, Na^+, K^+, HCO_3^- and glucose, as well as metabolic activity reflected by ammoniagenesis [92]. These findings, together with promising results observed in an AKI setting, paved the way towards the first clinical trials. These were performed in CKD and AKI patients using a renal assist device containing primary human renal tubule cells. Even though there was a remarkable beneficial impact on inflammatory status, metabolic activity and patient survival, the treatment and further development of the device were discontinued due to safety reasons related to low platelet counts and difficulties with the manufacturing process [93,94]. Another development in the field of cell-based renal assist and BAK devices was the so-called Bioartificial Renal Epithelial Cell System (BRECS), a perfusion bioreactor composed of primary renal epithelial cells derived from progenitor cells with proven renal function and grown on porous, niobium-coated carbon disks. BRECS was designed to offer various advantages, including immunoisolation, and especially to enable cryopreservation, thus allowing mass production of the system [95]. In addition, there is also the next-generation BAK device, a wearable BAK (WEBAK) that seeks to implement both tubular function of cells and filtration function of conventional hemodialysis [65]. From the composition point of view, the

WEBAK makes use of sorbent-based dialysis and the BRECS system as mentioned earlier, both of which contribute to the device wearability. By being able to adsorb and trap various solutes from effluent dialysate, sorbent materials allow its purification and regeneration and therefore reduce the required volumes of dialysate solutions as compared to conventional systems [96]. The BRECS on the other hand, by containing renal epithelial cells, should be able to replace kidney tubule function [95].

CELLS FOR EFFICIENT BAK APPLICATION

Despite all advances in the field of cell-based renal replacement therapies, no successful clinical applications have been reported so far. This is in part due to the fact that the protein-bound uremic toxins are not actively secreted by the cell types that were subject of those studies. It is clear that for safety reasons and probably different requirements for growth and differentiation, animal-derived cell lines such as MDCK and Lilly Laboratories Cell Porcine Kidney proximal tubule cells (LLC-PK_1) are not preferred for such applications. Even though the activity of the OCT2 was shown for LLC-PK_1, there was no or minimal expression of other crucial transporters involved in the uptake or excretion of uremic waste metabolites, such as BCRP or P-gp [97-100]. From a safety and physiological relevance perspective the preferred cell type for BAK would be primary human PTEC. However, main drawbacks of these cells are the relatively scarce availability, the high variability between donors, especially in terms of function, and a rapid loss of relevant transporters essential for uremic toxin clearance during expansion and culture [101,102]. Moreover, human renal and progenitor cells could represent another possible cell source for BAK use, as described previously [103-105]. However, other than proven polarization of cells with the expression of aquaporin-2 and Na^+K^+ATPase pump, no expression and function of other transporters, required for active secretion of waste metabolites, was demonstrated [103]. Another possible solution is given by human PTEC with extended lifespan using antisense oligonucleotides or RNA interference to suppress gene expression of cell cycle-related genes, such as pRb, p53, $p21^{Cip1}$ and $p16^{INK4a}$ [106]. Despite demonstrated reabsorption activity and beneficial effect on inflammatory status, the active secretion of uremic metabolites and specific transporters expression have not been addressed [106,107].

As mentioned before, some of the most important transporters necessary for the active transport and removal of xenobiotics and endogenous waste products are the OCT2, OAT1 and OAT3, which are located at the basolateral membrane of PTEC and are responsible for the uptake of organic cations and anions, respectively. On the other hand, the most important efflux proteins are MATE1, MATE2, P-gp, MRP2, MRP4 and BCRP [9,13,108].

A promising solution to the observed drawbacks of most cell types investigated for BAK purposes is provided by conditionally immortalized PTEC (ciPTEC) [109]. The conditional immortalization of these cells, mediated by retroviral transduction of a temperature-sensitive mutant of Simian virus 40 Large T antigen (SV40T), U19tsA58, and of a catalytic subunit of human telomerase (hTERT), allows the expansion and availability, while maintaining stability and function [109]. In addition to the demonstrated expression of almost all relevant transporters

and efflux pumps mentioned earlier, the overexpression of OAT1 greatly increases the suitability of these cells for BAK use [110-113]. A recent study has shown that, when cultured on the polyethersulfone-based hollow fiber membranes, coated with L-3,4-dihydroxyphenylalanine (L-DOPA) and collagen IV, ciPTEC form a strong epithelial barrier and mediate transepithelial transport of two prototypical protein-bound uremic toxins – indoxyl sulfate and kynurenic acid [114]. In addition, endocytosis-mediated albumin uptake was also confirmed, suggesting a promising future application in a BAK device mimicking all major tubular functions.

GUIDELINES FOR SAFETY EVALUATION OF CELL-BASED THERAPEUTICS FOR CLINICAL APPLICATION

Cell-based therapies, such as BAK device for CKD treatment, are of high interest and hold great promise for many complex diseases. However, focus is often put on the developmental aspects, characterization of the function, and efficacy, rather than on safety issues. There are several guidelines currently valid in Europe and USA that offer, even though not complete, very useful recommendations and considerations on how to investigate and determine the safety related parameters of gene and cell-based products intended for medical and therapeutic applications. The following description of the safety and risk assessment of cell- and gene-based therapies is compiled from the official guidelines of the World Health Organization (WHO) [115], Food and Drug Administration (FDA) [116], and European Medicine Agency (EMA) [117], and it should be taken as minimal requirements for non-clinical examination before first use in human subjects.

Some of the general safety recommendations are related to the development of the cell type intended for therapeutic applications. Such cells can be autologous, allogeneic or even xenogeneic. For that reason, first of all, it is necessary to determine the authenticity and identity of the cells by genotypical and phenotypical characterization, and to confirm the absence of contaminations with another cell line. Karyology and isoenzyme analysis can help to determine the species of origin. However, DNA profiling of short tandem repeats (STRs) and multiple single nucleotide polymorphisms (SNPs), or Human Leukocyte Antigen (HLA) typing, are preferred in case of human-derived cells as this allows the creation of a profile specific to the individual from whom the cells were derived. Next to this, stability and functional integrity of cells over time is another key aspect of risk assessment. This is of particular interest as genetic instability can affect the function and, therefore, the quality and safety of the cell product. Even more when the cells are genetically modified to stably express exogenous DNA sequences. Therefore, the copy number of the construct and transgene used, as well as the sites of chromosomal insertion should be determined. This could be achieved by sequencing methods and *in situ* hybridization and it can help determining whether there are abnormal fusions with endogenous genes or events of insertional mutagenesis. This is extremely important as it can also have an effect on activation of latent viruses (herpes zoster, cytomegalovirus and Epstein-Barr virus).

With regard to previously mentioned points, the tumorigenic effect of cells should be carefully evaluated, especially if cells underwent genetic manipulation procedures. The guidelines suggest assessing tumorigenic phenotype by inoculating cells in test animals that are deficient in cytotoxic

T-lymphocyte activity as to avoid xenograft rejection. Genetically immunocompromised rodents, such as nude mice and severe combined immunodeficient (SCID) mice, are among the most used test animals. Cells that are intended for evaluation of tumorigenicity should be propagated to the proposed *in vitro* cell age used for production or beyond, and a minimum of 10^7 cells should be inoculated either intramuscularly or subcutaneously to animals that should be observed for tumor formation and metastases for at least 4 months. Besides, oncogenic potential, defined as the property of an acellular agent (DNA, oncogenic viral agents, etc.) to induce cells of an animal to become tumor cells, should be examined as well. This is usually done by inoculating ≥100 μg of DNA or lysates derived from 10^7 cells and prepared by a method that ensures cell lysis, while avoiding disruption of eventually present viral particles. As briefly mentioned earlier, genetic stability is extremely important, especially with genetically modified cells. Therefore, a careful cytogenetic analysis, by karyotyping, should be performed regularly.

Furthermore, when cells have to be transplanted, it is important to assess several parameters indicative of cell fate *in vivo*, including their survival and distribution. To this end, various imaging techniques can be employed depending on cell labelling (fluorescence, iron-dextran nanoparticles, radioisotope etc.), as well as PCR analysis. Moreover, if cells used are not completely differentiated or are derived from induced pluripotent stem cells (iPSC), it is desirable to assess differentiation capacity and transdifferentiation that may be influenced by their microenvironment and, thus, have an impact on their biologic activity or safety, such as ectopic expression in non-target tissues. For that reason, similarly to what has been suggested for tumorigenic studies, it is essential to validate cell level of differentiation *in vivo*.

Another critical point to consider when determining risk factors of cell-based therapies is a potential immunological response directed against either the scaffold or the cellular component of a device. In particular, if cells are of allogeneic or xenogeneic origin there might be an unwanted immune response to those cells and, therefore, aspects of humoral and cell-mediated immunity should be determined.

In relation to genetically modified cells, and depending on the vectors and plasmids used, it is necessary to examine the release of vectors *in vivo*, especially because there could be undesired interactions with other infectious agents or unwanted immune reactions. In addition, eventual dissemination of vectors to several tissues and organs such as gonads should be addressed as it could affect or modify the germline due to integration and insertional mutagenesis.

A final important point that has to be ascertained is the absence of microbiological contaminations. As cells are subject to contaminations and have the ability to propagate microorganisms, such as mycoplasma and viruses, it is crucial to extensively test cells prior to any clinical application. To this end, various approaches can be employed depending on the microorganism that has to be detected. As for viruses, there are several methods to be used ranging from *in vivo* experiments in mice, guinea-pigs, rabbits, embryonic chicken eggs, etc. to transmission electron microscopy (TEM) and specific tests for retroviral particles, including quantitative PCR-based reverse transcriptase (RT) assay or product-enhanced reverse transcriptase (PERT) assay. When RT assays result positive, infectivity assays using cells that can support retroviral growth should be employed to show whether identified viruses are replicative. Furthermore, tests for

bacterial contaminations should be performed as well, such as inoculation of cell samples in specific growth media supporting bacterial proliferation or specific molecular methods based on PCR. For maximum control and prevention of contamination, all materials used for cell culture and expansion, like cell culture medium, should be free of any animal components.

Yet, it should be noted that all recommendations mentioned here are not definite and their use should be carefully evaluated and adjusted depending on the cell type, genetic modification, desired way of application such as transplantation, implantation or as extracorporeal medical device, and finally the type of disease that is meant to treat.

AIM AND OUTLINE OF THE THESIS

Despite the availability of several treatment modalities for CKD, including kidney transplantation and dialysis, mortality within this patient population is still rather high, underlining existing limitations of current renal replacement therapies. Hence, it is not unexpected that innovative therapeutic options for CKD have been the subject of extensive studies in recent years. Such promising applications, including BAK and other cell-based devices, have shown great potential and encouraging *in vitro* results were obtained from a functional point of view. However, safety issues and risk assessment of the cellular component of such products are often undervalued and not evaluated promptly.

The aim of this thesis was to investigate in a preclinical setting the safety aspects of the genetically modified ciPTEC line for BAK application. Following an introduction on kidney disease, current needs for improved treatment and guidelines to assess safety of cell-based medicinal products, **Chapter 2** presents a systematic review of the existing literature of animal studies involving safety evaluation of genetically modified cells for kidney disease treatment. Various safety-related outcome measures were evaluated in several relevant animal models to determine the possible side effects of cell-based therapy. Also, the main drawbacks and limitations of available studies regarding risk assessment are discussed. In **Chapter 3** the evaluation of ciPTEC ability to produce the active form of vitamin D, 1α,25-dihydroxy-vitamin D_3, is presented. This is an important endocrine function of kidney proximal tubule cells that is severely affected or missing in CKD patients, and cannot be replaced by standard dialysis treatment options other than supplementation. Furthermore, the beneficial and protective effect of the active form of vitamin D on ciPTEC function and behavior in uremic conditions is examined. Continuing further with safety related issues, the immunogenic effect of ciPTEC lines was assessed and presented **in Chapter 4**. Considering the allogeneic origin of ciPTEC, cells were extensively characterized for the expression of various antigens such as HLA, as well as production of immune and inflammatory mediators. Finally, the interaction with immune cells was determined *in vitro* to give more insight into ciPTEC allostimulatory potential. **Chapter 5** describes the development of an up-scaled prototype BAK device and ciPTEC behavior in that setting. In particular, cell barrier and transport functions were examined, as well as production and release of inflammatory and immunomodulatory mediators in relation to device constitution. Taking into account the genetic modifications that ciPTEC underwent in order to create a readily available cell source for future BAK application, the safety aspects of retrovirally-

mediated conditional immortalization were extensively studied and the results are presented in **Chapter 6**. In particular, cell behavior concerning proliferation, apoptosis-resistance, migration, invasion and other features of transformed and cancer-like cells were explored. Genetic stability and insertional mutagenesis effects pertinent to transgenes integration were also evaluated. Finally, an *in vivo* study was performed to assess the tumorigenic and oncogenic potentials of ciPTEC. **Chapter 7** presents a comprehensive discussion and implications of the findings described in this thesis, as well as future perspectives in the context of BAK development. Finally, **Chapter 8** offers a summary of the presented work.

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Invited for submission in Pharmacology & Therapeutics

CHAPTER

ARE CELL-BASED THERAPIES FOR KIDNEY DISEASE SAFE? A SYSTEMATIC REVIEW OF PRECLINICAL EVIDENCE

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ABSTRACT

The number of individuals affected by acute kidney injury (AKI) and chronic kidney disease (CKD) is constantly rising. In light of the limited availability of treatment options and their relative inefficacy, cell based therapeutic modalities have been studied. However, not many efforts are put into safety evaluation of such applications. The aim of this study was to review the existing published literature on adverse events reported in studies with genetically modified cells for treatment of kidney disease.

A systematic review was conducted by searching PubMed and EMBASE databases for relevant literature, using pre-defined criteria. After initial screening of 4061 abstracts, a total number of 49 preclinical studies was finally included for full assessment. Of these, 34 (69%) presented inappropriate study design for safety parameters evaluation, only 2 studies (4%) had the optimal study design, while 13 (27%) showed sub-optimal study design with either direct or indirect evidence of adverse events. The high heterogeneity of included studies regarding cell type and number, genetic modification, administration route, and kidney disease model applied, combined with the consistent lack of appropriate control groups, makes a reliable safety evaluation of kidney cell-based therapies impossible. Only a limited number of included relevant studies looked into essential safety-related outcomes, such as immunogenic, tumorigenic and teratogenic potential, cell biodistribution, microbial safety with respect to microorganism contamination and latent viruses reactivation, as well as overall well-being and animal survival.

Given the fast development and increasing interest in advanced cell-based therapies to treat kidney disease, it is clear that showing efficacy is not sufficient to proceed with eventual clinical applications. For that reason, well-designed preclinical studies, including all required control groups and good manufacturing processes securing safety, need to be done early in development. Preferably, this should be performed side by side with efficacy evaluation and according to the official guidelines of leading health organizations, to reveal the most optimal parameters that could guarantee safety of kidney cell therapies and encourage further research towards efficient clinical applications.

Keywords: systematic review; cell therapy; genetically modified cells; side effects; risk factors; biodistribution; tumorigenicity; immunogenicity; kidney disease

INTRODUCTION

Renal insufficiency represents an important health problem and there is a great need for development of new treatment options that could improve quality of life of kidney patients, but also reduce the global social and economic burden on the healthcare system [1]. More awareness needs to be raised for prevention of acute kidney injury (AKI) [2] to reduce the mortality and the risk of developing chronic kidney disease (CKD). Also, more effort has to be put into improving the treatment of CKD itself and its progression towards end stage renal disease (ESRD) [3,4]. For ESRD, the preferred treatment option is kidney transplantation, but this is severely

limited by donor organ shortage and complications of immunosuppressive therapy. Therefore, the currently most frequently used therapy for ESRD is hemodialysis [5]. Despite many technological developments and advancements introduced in the field of dialysis in the past few decades, noticeable improvements regarding clinical outcomes, in particular patient survival, are still absent [6]. Dialysis therapy might reduce progression of the disease, however, it does not efficiently remove metabolic waste products, leading to their accumulation. These so called uremic toxins have been associated with the development of other co-morbidities over time, especially cardiovascular disease, which remains the main cause of death within CKD and ESRD populations [7-9]. In addition, the treatment sessions are cumbersome for patients, reducing noticeably their quality of life [10,11], further indicating the need for alternative treatment strategies.

Tissue engineering and regenerative medicine represent the extremely promising innovative strategies that could improve or replace functions of damaged organs, including the kidney, or even repair and regenerate them [12]. One of the recent developments in the field of tissue engineering includes the bioartificial kidney, comprised of viable epithelial cells of either allogeneic or xenogeneic origin, to make use of their transport machinery for a more efficient excretion of waste molecules [13-16]. On the other hand, regenerative medicine related approaches might make use of acellular components, such as synthetic biomaterials and scaffolds, or decellularized kidneys that would maintain the complex 3D organization of extracellular matrix (ECM), thus allowing the optimal growth and differentiation of the cells, in particular stem cells [12,17-19]. Alternatively, the stem cells could also be used directly by transplantation, without using scaffold materials, completely relaying on their regenerative capacities, including beneficial paracrine effects [17] .

The most frequently used cell types for cell-based CKD treatment are mesenchymal stem cells (MSC), bone marrow cells (BMC), endothelial progenitor cells (EPC) and hematopoietic stem cells (HSC) [20]. However, other cells types have also been studied for their potential to treat kidney disease, such as primary kidney cells [16,21], cell lines [22] or cells genetically modified to overexpress certain proteins with therapeutic effects, such as IGF-1 [23], HGF [24], EPO [25], CXCR4 [26,27], SAA1 [22,28,29], kallikrein [30], VEGF [31], and BMP-7 [32]. In addition, recent advances in the field of induced pluripotent stem cells (iPSC) as cell-based therapies for various pathologies, make them a very promising clinical approach and a valuable therapeutic tool for kidney failure as well [33,34].

In various animal models of CKD, many of these treatment options were shown to be beneficial, as evaluated by improvement of several histological outcomes (glomerulosclerosis and tubular interstitial fibrosis), as well as functional parameters (glomerular filtration rate (GFR), blood pressure, urinary protein, plasma urea, plasma creatinine). Experimental models of CKD included ischemia-reperfusion injury, diabetic nephropathy, subtotal nephrectomy, hypertension or drug induced kidney disease [20].

Despite these promising findings of cell-based therapy efficacy for CKD and ESRD, most studies have focused primarily on functional readouts and improvement of clinical parameters. An often neglected aspect and concern encountered when developing and improving cell-based therapies, is safety of the clinical application and especially the long-term effects.

Several safety issues related to cell-based therapies should be addressed prior to clinical application [35,36]. According to the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA), among the most relevant issues are immunogenic, immunotoxic and inflammatory effects, especially in case of allogeneic and xenogeneic cells. Furthermore, immortalized or cells modified and transduced with retro- or lentiviral vectors are of particular concern given the possibility of release of vectors or plasmids. In addition, such modifications could significantly affect cell morphology and behavior, strongly associated with transformed phenotype and oncogenic and tumorigenic potential. Presence of microbial agents is another not negligible issue to be taken into consideration when evaluating the safety of a specific cell type for clinical applications. A systematic review of the evidence on safety of genetically modified cells *in vivo* would provide important information for future studies aiming to assess possible therapeutic effects and safety of cell-based therapies for kidney disease. Since no such synthesis of evidence currently exists, we have performed a systematic review of all published preclinical evidence on the safety of immortalized and genetically modified cells in animal models of kidney disease. We investigated *a)* whether studies using genetically modified cells in animal models of kidney disease were set up to identify adverse outcomes or safety concerns and *b)* which safety concerns or adverse outcomes were reported.

MATERIALS AND METHODS

Review protocol and amendments

The review methodology was pre-specified in a review protocol and registered on http://www.syrcle.nl (see also [37]). We made the following amendments to the review protocol: the protocol has been extended on all genetically modified cells in addition to immortalized cell lines. The review question was defined as: 'What is the current evidence for the safety of cell therapy using immortalized and genetically modified cells in animal models of kidney disease?'

Literature search

A systematic review was performed of all available studies reporting safety and adverse effects evaluation of cell-based therapies, in particular immortalized and genetically modified cells, in various animal models of kidney disease. A systematic literature search for articles published up to March 2015, was performed in PubMed and EMBASE databases. The full search strategies for PubMed and EMBASE are included in Table 2.S1, and involved the following components: "cell and tissue based therapy", "kidney disease", and "animals". Articles obtained by this search were selected independently by two researchers based on title and abstract screening and according to inclusion and exclusion criteria. If abstracts were not available or not informative enough, the full-text article was screened. In case of discrepancies between the two independent reviewers, a third investigator was involved in the screening and discussion in order to reach a decision regarding inclusion. No language restrictions were applied. If needed, non-English articles were translated by native speaking scientists.

Inclusion and exclusion criteria

Articles were included if they were primary studies presenting unique data on *in vivo* experiments in which animals were treated with genetically modified cells to treat renal disease and any outcome related to safety or adverse events was reported.

Articles that met at least one of the following exclusion criteria during the title and abstract screening phase were not included: (1) there was no kidney disease, (2) there was no cell therapy intervention, (3) the study was not performed in animals *in vivo*, (4) immunodeficient animals were used and (5) the study was not a primary study. After initial screening, based on title and abstract, all articles included were subjected to full-text screening with additional exclusion criteria: (6) no genetically modified or immortalized cells were administered, (7) there were no safety related outcomes measured or indicated and (8) the full-text article was not retrievable.

Data extraction and analysis

The following study characteristics were extracted from all articles included: animal species, strain, age, sex, weight, kidney disease model, induction of kidney disease, cell type used for therapeutic intervention, cell origin, type of genetic modification of cells, number of cells administered, and administration route. Bibliographic data, such as author and year of publication, were also registered. The following outcomes related to safety and adverse effects were extracted: inflammatory and immune related markers (gene and protein cytokine levels, inflammatory cell infiltration), renal fibrosis (interstitial fibrosis, total collagen, gene and protein expression of TGF-β, α-SMA, PDGF, type I collagen and matrix metalloproteinases), apoptosis (TUNEL, caspase 3 activity and expression, Bax and Bcl2 expression), organ and tissue distribution of cells (kidneys and other distant organs), tumor and teratoma formation, and overall survival of animals.

Due to the high heterogeneity in study characteristics and in reporting of outcomes among all articles included, a meta-analysis was not possible. Therefore, a narrative synthesis of available data was performed.

RESULTS AND DISCUSSION

Study selection and characteristics

The electronic search strategy retrieved 987 articles from PubMed and 3623 articles from EMBASE, of which 4061 were unique and evaluated for inclusion based on title and abstract. In total, 287 articles met the inclusion criteria and were screened as full-text. After full-text assessment, 49 studies were included in this systematic review (Figure 2.1).

Study characteristics of all included articles are summarized in Tables 2.1 and 2.2, reporting animal and kidney disease models, as well as data on cell therapy and measured outcomes related to safety and adverse events. There was considerable variation in the characteristics of the animals and the renal disease models employed. 23 studies (46.9%) were performed in rats, 21 (42.9%) in mice, 2 (4.1%) in goats, 2 (4.1%) in rabbits and 1 (2.0%) in pigs. Next, regarding the sex of the animals used, 22 studies (44.9%) employed animals of male sex, 16 (32.7%) used female

animals, 4 (8.2%) used both sexes, while 7 studies (14.3%) did not specify the sex of animals. As far as it concerns renal disease models, there were 27 studies (55.1%) using an AKI model (either ischemia-reperfusion (I/R), cisplatin, gentamicin, nephrectomy or sepsis-induced), 7 (14.3%) using a CKD model (either a subtotal nephrectomy or unilateral ureter obstruction (UUO)), 5 (10.2%) a glomerulosclerosis model (both Alport syndrome and other models), 2 (4.1%) cystinosis, 2 (4.1%) a glomerulonephritis, 2 (4.1%) a diabetic nephropathy, 1 (2.0%) a polycystic kidney disease model, 1 (2.0%) a kidney transplantation model, 1 (2.0%) adriamycin nephrosis model and 1 (2.0%) study using both AKI and CKD models. Regarding cell types used a majority of studies (40; 81.6%) used some type of stem cells as indicated in Table 2.2, 7 studies (14.3%) used primary cells (renal tubular cells, macrophages or endothelial cells) and only 2 studies (4.1%) used cell lines. The number of administered cells was also variable with 3 studies (6.1%) using a cell number in the order of 10^8, 4 (8.2%) in the order of 10^7, 25 (51.0%) in the order of 10^6, 10 (20.4%) in the order of 10^5, 6 (12.2%) used 2 or more different amounts ranging from 10^3 to 10^7, while 1 (2.0%) study did not specify the number of administered cells.

All included articles were subdivided in one of four categories based on study design, whether appropriate controls were used in order to determine side effects and risk factors, as well as whether such adverse effects were reported explicitly and characteristics described. Description of all categories and categorized articles is shown in Figure 2.2a-b.

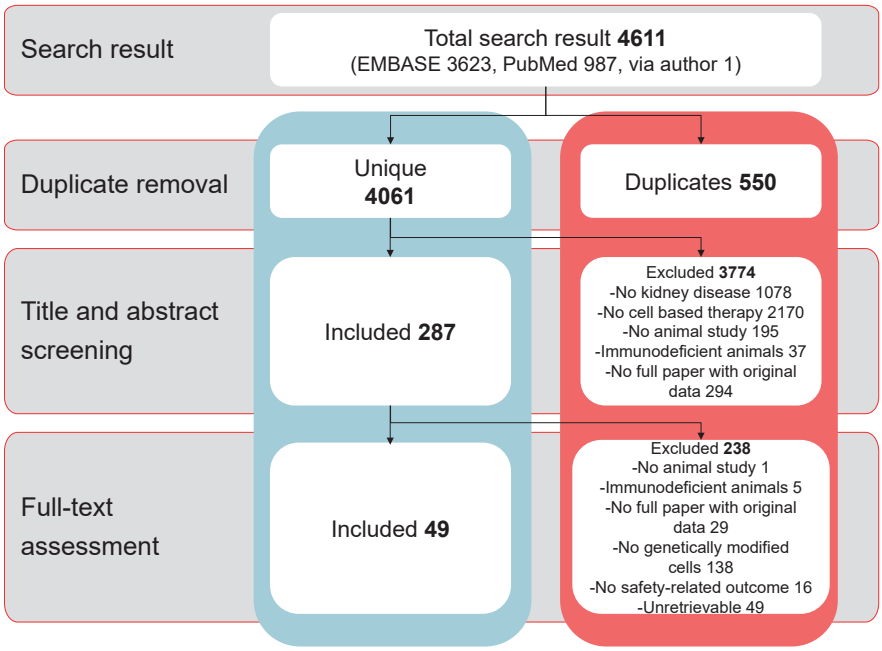


Figure 2.1. Flow chart of study selection. Complete search strategy is described in Table 2.S1. Inclusion and exclusion criteria used for articles selection are defined in the Material and Methods section.

Table 2.1. Study characteristics: Animals and kidney disease models.

Author and Year	Species	Strain	Age (weeks)	Sex (M/F)	Weight (g)	Kidney disease model	Induction of kidney disease
Baulier *et al.* 2014	Pig	NR	12	NR	NR	Kidney transplantation	Renal pedical clamping for 60 min followed by kidney removal, preservation and transplantation 6 days later
Bian *et al.* 2014	Rat	Sprague Dawley	Adult	M	200-250	CKD	Subtotal 5/6 nephrectomy
Chen *et al.* 2008	Mouse	FVB/NJ	8 to 20	M	NR	AKI (I/R)	Unilateral or bilateral renal pedicle clamping for 30 min
Chen *et al.* 2011	Rat	Sprague Dawley	Adult	F	200-250	AKI (I/R)	Bilateral renal pedicle clamping for 60 min
Eliopoulos *et al.* 2011	Mouse	BALB/c	8 to 10	F	NR	AKI	Subcutaneous cisplatin injection (14.7 mg/kg)
Ferenbach *et al.* 2010	Mouse	FVB/NJ	6 to 8	M	NR	AKI (I/R)	Left renal pedicle clamping for 20 min
Franchi *et al.* 2014	Rat	Sprague Dawley	6	F	NR	PKD	Rat PKD model [38]
Furuichi *et al.* 2012	Mouse	C57BL/6J	6 to 8	M	NR	AKI (I/R)	Left renal artery and vein clamping for 45 min
Gao *et al.* 2012	Rat	Sprague Dawley	NR	M	180-200	AKI (I/R)	Renal pedicles clamping for 40 min
Gheisari *et al.* 2012	Mouse	BALB/c	8 to 12	M	20-25	AKI	Subcutaneous cisplatin injection (18 mg/kg)
Hagiwara *et al.* 2008	Rat	Wistar	NR	M	NR	AKI (I/R)	Renal pedicles clamping for 40 min
Han *et al.* 2013	Mouse	C57BL/6	8	M	27-32	AKI (I/R)	Renal pedicles clamping for 30 min
Harrison *et al.* 2013	Mouse	C57BL/6	4 to 16	M/F	NR	Cystinosis	Cystinosis transgenic model (CTNS-/-)
Huang *et al.* 2012	Rat	NR	NR	NR	NR	Glomerulo-nephritis	Nephrotoxic serum nephritis induced by administration of rabbit antibodies against glomerular basement membrane
Imberti *et al.* 2007	Mouse	C57BL/6J	8	F	NR	AKI	Subcutaneous cisplatin injection (12.7 mg/kg)
Katayama *et al.* 2008	Mouse	129X1/SvJ	3	F	NR	Alport syndrome (glomerulo-sclerosis)	Alport syndrome transgenic model
Kelley *et al.* 2013	Rat	ZSF1	18	M/F	NR	Diabetic nephropathy	ZSF1 rat diabetic nephropathy model
Kelly *et al.* 2010	Rat	Sprague Dawley	NR	NR	250	AKI	Renal pedicles clamping for 30 min; gentamicin (100 $mg \cdot kg^{-1} \cdot day^{-1}$ twice daily for 7 days; cisplatin (7.5 mg/kg)
Kelly *et al.* 2012	Rat	Sprague Dawley	NR	F	150-200	AKI (I/R); CKD	Renal pedicles clamping for 50 min; intraperitoneal cisplatin injection (1.5 mg/kg every other day, 3 doses)

Author and Year	Species	Strain	Age (weeks)	Sex (M/F)	Weight (g)	Kidney disease model	Induction of kidney disease
Kelly *et al.* 2013	Rat	Sprague Dawley	10	F	NR	CKD	Clamping renal pedicles for 25 min in diabetic rat model
Kinomura *et al.* 2008	Rat	Sprague Dawley	NR	M	200-250	AKI	Intraperitoneal cisplatin injection (6 mg/kg)
Kucic *et al.* 2008	Mouse	C57BL/6	NR	F	NR	CKD	Electrocoagulation right kidney, followed by left kidney nephrectomy after 22 days
LeBleu *et al.* 2009	Mouse	C57BL/6, 129Sv	5 to 8	F	NR	Alport syndrome (glomerulo-sclerosis)	Alport syndrome transgenic model
Lee *et al.* 2012	Rat	Sprague Dawley	8	M	300	AKI (I/R)	Left renal artery clamping for 45 min
Li *et al.* 2006	Rat	Sprague Dawley	10	NR	NR	Glomerulo-sclerosis	Intravenous injection of anti-rat Thy1 monoclonal antibody (1 mg/kg/body weight)
Li *et al.* 2006(2)	Mouse	BALB/c	6	M	20-25	Adriamycin induced nephrosis	Intravenous adriamycin injection (10.5 mg/kg) 12 weeks after cell transplantation
Li *et al.* 2012	Mouse	B6-Ly5.2	6 to 8	F	NR	AKI (I/R)	Left renal pedicles clamping for 35 min
Lin *et al.* 2003	Mouse	B6-Ly5.2/Cr	6	F	NR	AKI (I/R)	Left renal artery clamping for 15 min
Liu *et al.* 2011	Rat	Sprague Dawley	6	F	140-160	CKD (UUO)	Left ureteral double ligation
Liu *et al.* 2013	Mouse	C57BL/6J	6 to 8	NR	NR	AKI (I/R)	Bilateral renal pedicles clamping for 30 min
Luo *et al.* 2014	Mouse	C57BL/6	NR	M	25-30	AKI (sepsis associated)	Cecal ligation and puncture (CLP)
Lv *et al.* 2014	Rat	Wistar	NR	F	200-250	Diabetic nephropathy	Intraperitoneal streptozotocin injection (60 mg/kg)
Pacurari *et al.* 2013	Rat	Sprague Dawley	11	M	NR	CKD	Subtotal 5/6 nephrectomy
Prodromidi *et al.* 2006	Mouse	C57BL/6	6 to 8	F	NR	Alport syndrome (glomerulo-sclerosis)	Alport syndrome transgenic model
Rampino *et al.* 2011	Rat	Sprague Dawley	NR	M	180-200	Glomerulo-sclerosis	Intravenous injection of anti-rat Thy1 monoclonal antibody (0.4 mg/rat)
Ruan *et al.* 2013	Rabbit	Japanese white rabbits	12	NR	2000-2600	CKD (UUO)	Left ureteral ligation
Saito *et al.* 2012	Goat	Japanese Saanen	NR	M	32000-50000	AKI	Bilateral nephrectomy and LPS injection ($5x10^5$ IU/kg) for 2h
Shuai *et al.* 2012	Rat	Sprague Dawley	NR	F	180-200	CKD	Subtotal 5/6 nephrectomy
Syres *et al.* 2009	Mouse	C57BL/6	8 to 16	M/F	NR	Cystinosis	C57BL/6 Ctns-/- transgenic mouse model

Author and Year	Species	Strain	Age (weeks)	Sex (M/F)	Weight (g)	Kidney disease model	Induction of kidney disease
Takahashi *et al.* 2013	Goat	Japanese Saanen	NR	M	NR	AKI	Bilateral nephrectomy, LPS for 2h during circulation
Togel *et al.* 2008	Mouse	C57BL/6	Adult	M/F	20-25	AKI (I/R)	Renal pedicles clamping for 30 min
Togel *et al.* 2009	Rat	Sprague Dawley, F344	Adult	M	200-300	AKI (I/R)	Renal pedicles clamping for 58, 40 or 35 min
Togel *et al.* 2009(2)	Rat	Sprague Dawley	Adult	F	200-250	AKI (I/R)	Renal pedicles clamping for 48 min
Tsuda *et al.* 2010	Rat	Lewis	6	M	170-180	Glomerulo-nephritis	Intravenous injection of anti-rat Thy1 monoclonal antibody (0.5 mg/rat)
Tsuda *et al.* 2014	Rat	Lewis	10	M	230-250	AKI (I/R)	Renal pedicles clamping for 60 min
Wise *et al.* 2014	Mouse	C57BL/6J	6 to 8	M	NR	AKI (I/R)	Left renal pedicle clamping for 40 min or bilateral renal pedicles clamping for 25 min
Yuzeng *et al.* 2014	Mouse	C57BL/6	6	M	NR	AKI (I/R)	Bilateral renal pedicles clamping for 40 min
Zhen-Qiang *et al.* 2012	Rabbit	NR	NR	NR	NR	AKI (I/R)	Left renal artery and vein clamping for 60 min
Zhuo *et al.* 2013	Rat	Sprague Dawley	8 to 10	M	250-300	AKI (I/R)	Right renal artery and vein clamping for 60 min

Sex (M/F) – Sex male or female; Weight (g) – Weight in grams. CKD - Chronic Kidney Disease; AKI – Acute Kidney Injury; I/R – Ischemia Reperfusion Injury; PKD – Polycystic Kidney Disease; UUO - unilateral ureteral obstruction; CRF – Chronic Renal Failure; NR – Not Reported.

Table 2.2. Study characteristics: Cell therapy applied (cell type, origin, modification, number and administration route) and outcomes measured.

Author and Year	Cell type	Cell origin (species)	Type of genetic modification (Gene of interest)	Number of cells	Administration route	Outcomes
Baulier *et al.* 2014	afMSCs	Autologous	Lentiviral transduction (GFP)	$1x10^6$/kg body weight	Intrarenal artery	I, F, D
Bian *et al.* 2014	MSCs	Allogeneic	Lentiviral transduction (GFP)	$1x10^7$	Intravenous	F, D
Chen *et al.* 2008	4E	Allogeneic	Transgenic mouse (Tie-2/ GFP)	$1x10^6$	Intravenous	I, F, A, D
Chen *et al.* 2011	hucMSCs	Xenogeneic (human)	Adenoviral transduction (GFP, HGF)	$1x10^6$	Intracarotid artery	I, A, D, T
Eliopoulos *et al.* 2011	MSCs	Allogeneic	Retroviral transduction (EPO)	$5x10^6$	Intraperitoneal	A, D, S
Ferenbach *et al.* 2010	Macrophages	Allogeneic	Adenoviral transduction (HO-1)	$5x10^6$	Intravenous	I, A, D
Franchi *et al.* 2014	MSCs	Allogeneic	Transfection (Luciferase)	$2.5x10^5$	Intrarenal artery	F,D
Furuichi *et al.* 2012	ADMSCs	Allogeneic	Transgenic mouse (GFP)	$1x10^5$	Intravenous	I, F, D
Gao *et al.* 2012	ADMSCs	Allogeneic	Lentiviral transduction (Luciferase, RFP)	$2x10^6$	Kidney cortex	A, D
Gheisari *et al.* 2012	MSCs	Allogeneic	Lentiviral transduction (CXCR4, CXCR7, GFP)	$5x10^5$	Intravenous	A, D, S
Hagiwara *et al.* 2008	MSCs	Allogeneic	Adenovirus transduction (GFP, kallikrein)	$1x10^6$	Intracarotid artery	I, A, D
Han et al. 2013	MRPC	Allogeneic	Transgenic mouse (GFP)	$1x10^5$	Intravenous	I, D, T
Harrison *et al.* 2013	HSPCs	Allogeneic	Lentiviral transduction (GFP, Luciferase, CTNS, CTNS-GFP)	$1x10^6$	Intravenous	I, D, T
Huang *et al.* 2012	BM-MSCs	Xenogeneic (human)	Adenoviral transduction (GDNF-GFP)	$3x10^4$; $1x10^6$	Intrarenal artery	I, D
Imberti *et al.* 2007	MSCs	Allogeneic	Transfection (IGF1 siRNA)	$2x10^5$	Intravenous	D
Katayama *et al.* 2008	BMDCs	Allogeneic	Transgenic mouse (COL4A3 knock out)	$1x10^7$	Intravenous	F, S
Kelley *et al.* 2013	SRCs	Allogeneic	Transgenic mouse (Tomato red fluorophore reporter)	$2x10^6$	Parenchymal (kidney)	I, F, D, S
Kelly *et al.* 2010	NRK-52E	Allogeneic	Transfection (SAA1.1, GFP, reporter pNF-kB-SEAP)	$1x10^6$	Intravenous	I, A, D
Kelly *et al.* 2012	Renal tubule cells	Allogeneic	Transfection (GFP, SIRT1, SAA1, BFP)	$1x10^6$	Intravenous	I, F, A, D, S
Kelly *et al.* 2013	Renal tubule cells*	Allogeneic	Transfection (GFP, SAA1)	$1x10^6$	Intravenous	I, A, F, D
Kinomura *et al.* 2008	rKS56	Allogeneic	Transfection (β-galactosidase)	$1x10^6$	Subcapsular, intrarenal artery	A, D, T*

Author and Year	Cell type	Cell origin (species)	Type of genetic modification (Gene of interest)	Number of cells	Administration route	Outcomes
Kucic *et al.* 2008	MSCs	Allogeneic	Retroviral transduction (EPO, IGF-1)	$2x10^6$	Subcutaneous	A
LeBleu *et al.* 2009	BMDCs	Allogeneic	Transgenic mice (COL4A3 knock out, GFP)	$1x10^6$; $1x10^7$ (seven injections)	Retro-orbital	S
Lee *et al.* 2012	iPS	Xenogeneic (mouse)	Retroviral transduction (Oct-4, Sox2, Klf4, EGFP)	5×10^4; 1×10^5; 5×10^5; 5×10^6; 5×10^7	Intrarenal artery	I, A, D, T, S
Li *et al.* 2006	BMDCs	Allogeneic	Transgenic rat (EGFP)	$1x10^8$	Intravenous	I, D, S
Li *et al.* 2006(2)	BMDCs	Allogeneic	Transgenic mouse (EGFP)	$1x10^6$	Intravenous	F, A, D
Li *et al.* 2012	HSPCs (Lin^-)	Allogeneic	Transgenic mouse (Cre^{Ksp}:R26R-EYFP)	$5x10^6$	Intravenous	A, D, T
Lin *et al.* 2003	HSPCs (Lin^-)	Allogeneic / Autologous	Transgenic mouse (β-galactosidase)	$2x10^3$ (allogeneic) + $2x10^5$ (autologous)	Intravenous	I, A, D
Liu *et al.* 2011	MSCs	Allogeneic	Adenoviral transduction (GFP, HGF)	$1x10^6$	Intravenous	F, D
Liu *et al.* 2013	BM-MSCs	Allogeneic	Lentiviral transduction (CXCR4, GFP)	$2x10^6$	Intravenous	A, D
Luo *et al.* 2014	BM-MSCs	Allogeneic	Transgenic mouse (RFP)	$1x10^6$	Intravenous	I, D, S
Lv *et al.* 2014	MSCs	Allogeneic	Lentiviral transduction (GFP)	$2x10^6$	Intravenous	F, D
Pacurari *et al.* 2013	EC	Allogeneic	Adenoviral vector (GFP)	$1.5x10^6$	Intravenous	D
Prodromidi *et al.* 2006	BMDCs	Allogeneic	Transgenic mouse (COL4A3 knock out)	$1x10^7$	Intravenous	F, D
Rampino *et al.* 2011	MSCs	Allogeneic	Transgenic rat (EGFP)	$3x10^6$	Intravenous	I, F, D
Ruan *et al.* 2013	Fibroblasts (induced)	Autologous	Lentiviral transduction (EGFP)	$2x10^5$	Intrarenal artery	I, F, D
Saito *et al.* 2012	hRPTEC	Xenogeneic (human)	Transfection (p16INK4a siRNA)	$3\text{-}7x10^8$ / device	Extracorporeal circuit via jugular vein	I, S
Shuai *et al.* 2012	EPCs	Allogeneic	Transfection (TERT)	$1x10^5$	Intravenous	I, F, T
Syres *et al.* 2009	BMDCs	Allogeneic	Transgenic mice (GFP, Luciferase)	$2x10^7$	Intravenous	D
Takahashi *et al.* 2013	hRPTEC	Xenogeneic (human)	Transfection (p16INK4a siRNA)	$3\text{-}7x10^8$ / device	Extracorporeal circuit via jugular vein	I, S
Togel *et al.* 2008	MSCs	Allogeneic	Transfection (Luciferase)	$1x10^5$	Intravenous	A, D

Author and Year	Cell type	Cell origin (species)	Type of genetic modification (Gene of interest)	Number of cells	Administration route	Outcomes
Togel *et al.* 2009	MSCs	Allogeneic	Transgenic rat (hPAP)	$1.5x10^6$; $0.5x10^6$/kg; $2x10^6$/kg; $5x10^6$/kg body weight	Intracarotid artery	F, T, S
Togel *et al.* 2009(2)	MSCs	Allogeneic	Transfection (VEGF siRNA)	$2x10^6$/kg body weight	Intracarotid artery	S
Tsuda *et al.* 2010	FM-MSCs	Allogeneic	Transgenic mouse (GFP)	$5x10^5$	Intravenous	I, F, D
Tsuda *et al.* 2014	FM-MSCs	Allogeneic	Transgenic mouse (GFP)	$5x10^5$	Intravenous	I, F, D
Wise *et al.* 2014	MSCs	Xenogeneic (human)	NR (EGFP, Luciferase)	$1x10^6$	Intravenous	F, D
Yuzeng *et al.* 2014	MSCs	Allogeneic	Lentiviral transduction (Survivin, EGFP)	$1x10^6$	Intravenous	D
Zhen-Qiang *et al.* 2012	BM-MSCs	Allogeneic	Adenoviral transduction (hBMP7, GFP)	NR	Intrarenal artery	A, D
Zhuo *et al.* 2013	MSCs	Allogeneic	Lentiviral transduction (Luciferase, RFP)	$1x10^6$; $2x10^6$; $5x10^6$	Intravenous, intrarenal artery	D

I – Immune and Inflammation markers or related outcomes; F – Fibrosis related outcomes; A – apoptosis; D – Biodistribution; T – Tumor and teratoma; S – Survival; afMSCs - Amniotic fluid-derived mesenchymal stem cells; MSCs - Mesenchymal Stem/Stromal Cells; 4E - kidney-derived clonal cell line of MSC; hucMSCs - Human-umbilical cord-derived mesenchymal stem cells; ADMSCs - Adipose-derived mesenchymal stem cells; MRPC - Mouse renal progenitor cells; HSPCs - Hematopoietic stem and progenitor cells; BM-MSCs - Bone marrow-derived mesenchymal stem cells; BMDCs - Bone marrow-derived cells; SRCs - Selected renal cells; NRK-52E - Rat epithelial kidney cell line; rKS56 - Proximal tubule S3 segment-derived renal progenitor-like cell line; iPS – Induced pluripotent stem cells; EC – Endothelial cells; hRPTEC - Human renal proximal tubular epithelial cells; EPCs - Endothelial progenitor cells.

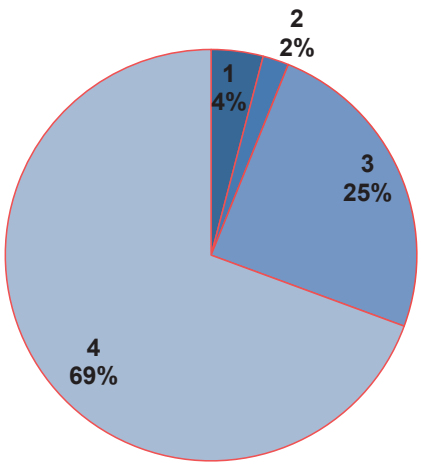
* Renal tubule cells - Mix of cells derived from proximal, ascending, collecting and distal tubules

T* - indirect evidence of tumorigenicity assessment (see Table 2.3)

(a)

Author and Year	Category	Author and Year	Category
Baulier *et al.* 2014	4	Li *et al.* 2006(2)	4
Bian *et al.* 2014	4	Li *et al.* 2012	3
Chen *et al.* 2008	4	Lin *et al.* 2003	4
Chen *et al.* 2011	1	Liu *et al.* 2011	4
Eliopoulos *et al.* 2011	4	Liu *et al.* 2013	4
Ferenbach *et al.* 2010	4	Luo *et al.* 2014	4
Franchi *et al.* 2014	3 -	Lv *et al.* 2014	4
Furuichi *et al.* 2012	4	Pacurari *et al.* 2013	3 -
Gao *et al.* 2012	4	Prodromidi *et al.* 2006	4
Gheisari *et al.* 2012	4	Rampino *et al.* 2011	3 -
Hagiwara *et al.* 2008	4	Ruan *et al.* 2013	4
Han *et al.* 2013	4	Saito *et al.* 2012	3 -
Harrison *et al.* 2013	3	Shuai *et al.* 2012	3
Huang *et al.* 2012	4	Syres *et al.* 2009	4
Imberti *et al.* 2007	4	Takahashi *et al.* 2013	4
Katayama *et al.* 2008	4	Togel *et al.* 2008	2
Kelley *et al.* 2013	3	Togel *et al.* 2009	3
Kelly *et al.* 2010	4	Togel *et al.* 2009(2)	3
Kelly *et al.* 2012	4	Tsuda *et al.* 2010	4
Kelly *et al.* 2013	4	Tsuda *et al.* 2014	4
Kinomura *et al.* 2008	3	Wise *et al.* 2014	4
Kucic *et al.* 2008	1	Yuzeng *et al.* 2014	4
LeBleu *et al.* 2009	4	Zhen-Qiang *et al.* 2012	4
Lee *et al.* 2012	3 -	Zhuo *et al.* 2013	4
Li *et al.* 2006	4		

(b)



(c)

	Wild-type cells		Genetically modified cells
No kidney disease	Healthy animals receiving wild-type cells	← → **Effect of genetically modified cells in healthy animals**	Healthy animals receiving genetically modified cells
	↑ **Effect of wild-type cells on kidney disease** ↓		↑ **Effect of genetically modified cells on kidney disease** ↓
Kidney disease	Animals with kidney disease receiving wild-type cells	← → **Effect of genetically modified cells in diseased animals**	Animals with kidney disease receiving genetically modified cells

Figure 2.2. Articles categorization based on suitability of study design and animal experimental set-up for determination of cell therapy risks and adverse events. Category 1 – Optimal study design (genetically manipulated and wild type cells administered in healthy control animals reporting adverse effects); Category 2 – Sub-optimal study design (genetically manipulated cells administered in healthy animals and monitoring for side effects); Category 3 – Sub-optimal study design with indirect evidence of adverse events (genetically manipulated cells administered only in disease animal models and reporting adverse events; studies indicated as 3- represent those that did not report explicitly adverse events, even though outcomes relevant to side effects were present); Category 4 – Inappropriate study design (genetically manipulated cells administered in disease animal models not reporting adverse events or relative outcome measurements). (**a**) List of included studies categorized as mentioned; (**b**) Percentages of studies divided over 4 categories. (**c**) Representation of an optimal study design in which both wild-type and genetically modified cells were used in healthy control animals and in animals with kidney disease. Such a study design would allow to determine both the efficacy and safety of a given type of genetically modified cells in certain model of renal disease.

The obtained results suggest that the majority of studies (69%) did not have appropriate control groups and did not report any adverse effects related to the cell therapy implemented to treat kidney disease. A suboptimal study design was found for 25% of included articles, presenting indirect evidences of cell therapy safety, specifically reporting adverse events during the course of the study. A total of 3 studies had the suitable study design allowing the assessment and monitoring of adverse effects. Only 1 study (2%) applied genetically modified cells in healthy control animals but did not evaluate specifically adverse events, while 2 (4%) were optimally designed and did monitor and report the side effects and cell therapy related problems. Finally, the optimal study design which would allow to determine both the efficacy and safety of a specific type of genetically modified cells is summarized in Figure 2.2c.

Kidney disease cell therapy-related adverse effects

Several risk factors and side effects of cell therapy that are generally recognized [39,40] (schematically shown in Figure 2.3) were considered in all included kidney disease animal studies. Most of the risk factors and side effects considered were related to stem cell based therapies, given the fact that stem cells were used by the majority of studies. Nonetheless, the information derived can be easily extrapolated to other cell types, especially genetically modified cells. However,

the description of various risk factors, including immunogenicity, tumorigenic effects, teratoma formation, biodistribution, microbial contaminations and overall well-being and survival, varied between studies depending on the cell type or genetic manipulation of the cells. Here, we discuss in more detail their relevance in perspective of the cells used in the selected articles.

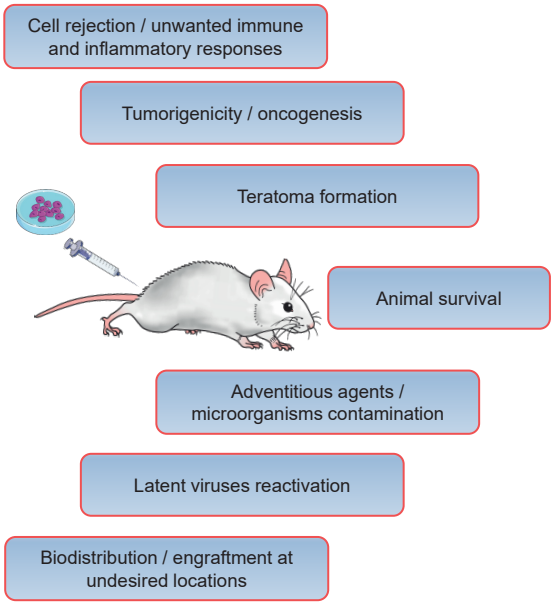


Figure 2.3. Possible risk factors and adverse events related to cell administration for therapeutic purposes.

Immunogenicity, cell rejection and tissue damage

Depending on their origin, especially for allogeneic and xenogeneic cells, it could be expected that cells undergo rejection, thus compromising cell therapeutic activity, or even elicit undesired inflammatory and/or immune responses. This is especially related to the expression of highly polymorphic major histocompatibility complex antigens (MHC), as extensively studied and demonstrated in the solid organ transplantation field [41]. Besides cell origin, several other parameters might influence immunogenicity of cell therapy, including the administration site, the need for multiple exposures or administrations, the effect of disease on immune system performance, as well as the maturation status of (stem) cells [42]. It has been reported that some cells, such as embryonic stem cells (ESCs), although immunologically immature because they lack MHC class II molecules expression are not completely resistant to immune rejection [43-46]. Considering all included studies, 3 used autologous cell transplantation, 6 used xenogeneic cells, while 40 studies described the use of allogeneic cells. Reported parameters that were taken into

account for possible assessment of inflammatory and immunogenic effects were predominantly cytokine expression and release, as well as inflammatory cell infiltration. In total, 24 studies were examined and showed at least one of the following outcomes: mRNA or protein serum levels of TNF-α, IL-6, IL-17, IL-10, IL-1α, IL-1β, MCP-1, IFN-γ, as well as leukocyte and ED-1 positive cells infiltration. Still, most of these findings were obtained as an indication of efficacy of cell therapy and attenuation of kidney disease with beneficial anti-inflammatory effects, rather than as a safety evaluation of the cell therapy. In addition, none of the studies examined systematically the expression of immune related antigens such as MHC Class I and II molecules or other co-stimulatory factors involved in immune system activation. From the observed data, it is not possible to infer any relevant conclusion concerning inflammatory and immune responses induced by specific cell types in kidney disease animal models, considering that there were no healthy or sham-operated animals treated with genetically modified cells and that the population of included studies was highly heterogeneous, especially in terms of animal species and renal disease models used, as well as specific cell type and their mode of application, as mentioned earlier and shown in Tables 2.1 and 2.2.

A total of 20 studies evaluated the presence of fibrotic markers such as mRNA or protein levels of TGF-β, α-SMA, PDGF, type I collagen and matrix metalloproteinases (MMP 2 and 9), as well as the extent of fibrotic tissue damage by trichrome periodic acid-Schiff or Sirius Red staining. Considering that fibrosis is a good indicator of tissue damage it could be a valid marker for inflammatory response related safety aspects as well, but also this parameter was almost exclusively addressed for therapeutic goals of cell therapy and to evaluate the impact on renal fibrosis progression.

Similar findings were observed in case of apoptosis, which was considered as a possible indicator of cell rejection, as suggested previously [47,48]. None of the 19 studies that reported any of the apoptosis-related markers, such as caspase 3 expression, DNA fragmentation (TUNEL staining), Bax and Bcl2 expression, had the optimal study design, or determined the apoptosis status of administered cells, other than reporting the protective effect on kidney tissue damage in terms of reduced number of apoptotic tubular cells. As discussed in later sections, several studies evaluated biodistribution and specifically persistence of cells in kidneys, which could be suggestive of cell survival and rejection following administration.

Tumorigenicity, oncogenicity and teratoma formation

Another important risk factor for cell-based therapies is the risk of tumorigenic and oncogenic effects, as well as teratoma formation. This is mostly due to the differentiation status of cells, or genetic manipulation and continuous cell culture which can lead to genetic aberrations and genomic alterations associated with cancer and tumor development [49-51]. Overall, 8 studies assessed teratoma, tumor or other malignant occurrences (Table 2.3). Kinomura *et al.*[52], who adopted Lac-Z transfected S3 segment-derived proximal tubule cells (rKS56), stated the tumorigenicity related findings indirectly (T*; Table 2.2) by referring to a previous study performed in nude mice, which suggested the absence of a tumor cell phenotype [53]. However, according to the karyotype analysis, they found that rkS56 cells employed as cell therapy were

nearly triploid regardless of LacZ transfection, and this could represent a risk of tumorigenesis [54,55]. The study by Chen *et al.* [24] assessed tumorigenic effect of hepatocyte growth factor (HGF) and GFP adenovirally transduced human umbilical cord-derived mesenchymal stem cells (hucMSC) in BALB/c nude mice, instead of in the kidney disease animal model. Their results indicated the absence of tumor formation in nude mice during a three-month observation period. Moreover, several other studies reported that tumor generation following cell administration into kidney disease animal models was not observed. For instance, Togel *et al.* [56] showed that MSCs generated from hPAP transgenic F344 rats did not give rise to tumors in a Sprague Dawley rat AKI model, while Han *et al.* [57] stated that there was no teratoma formation in kidneys of AKI C57BL/6 mice 6 weeks after injection of mouse adult renal progenitor cells derived from C57BL/6-GFP transgenic mice. Similarly, Li *et al.* [58] did not find any presence of teratoma in kidneys of mice with renal ischemic injury up to 6 months following injection of mouse hematopoietic stem and progenitor cells derived from a transgenic CreKsp:R26R-EYFP mice and induced to differentiate into cells resembling renal cell phenotype. Moreover, endothelial progenitor cells transfected with telomerase reverse transcriptase did not cause any malignant changes in chronic kidney disease setting [59]. Finally, Lee *et al.* [60] and Harrison *et al.* [61] showed in two different animal models of kidney disease that iPS cells reprogrammed with retroviral vectors encoding Oct-4, Sox2, Klf4 and EGFP, and hematopoietic stem and progenitor cells transduced with lentiviral vectors bearing CTNS and EGFP genes, respectively, did not induce tumor formation for the duration of the study. Overall, none of the studies that monitored animals for tumor and teratoma formation showed the undesired effects regardless of cell type and genetic manipulation adopted. However, when using genetically manipulated cells, as the studies included in this review have done, it is essential to evaluate genetic stability of the cells as this can be significantly compromised. Not only genetic modifications, such as transfection and transduction, but also the use of cells derived from transgenic donors and the regular expansion and culture of cells, can be associated with the introduction of chromosomal aberrations and, thus, increased risk of tumorigenicity in recipients [62,63]. Unfortunately, almost none of the studies included did perform a karyotype analysis or other assessment of genetic stability, despite dealing with genetically modified cells or cells derived from transgenic animals. However, it should be recognized that we excluded studies using immunodeficient animals, such as SCID mice, which are usually preferred for tumor xenograft evaluation. This was done because it was considered unjustified to combine data from immunodeficient and immunocompetent animals in a meta-analysis. Moreover, the kidney disease model in immunodeficient animals would not reflect the genuine pathophysiology.

Table 2.3. Tumor and teratoma formation assessment.

Author and Year	Species	Cell type	Tumor/teratoma formation outcome
Chen *et al.* 2011	Rat	hucMSCs	No tumor formation observed in nude mice during 3 months follow-up
Han *et al.* 2013	Mouse	MRPC	No teratoma formation observed during 6 weeks follow-up
Harrison *et al.* 2013	Mouse	HSPCs	No tumor formation observed during 12 months follow-up
Kinomura *et al.* 2008	Rat	rKS56	* Kitamura *et al.* [53]: No tumor formation observed in nude mice during 13 months follow-up
Lee *et al.* 2012	Rat	iPS	No tumor formation observed during 6 months follow-up
Li et *al.* 2012	Mouse	HSPCs (Lin^-)	No teratoma formation observed during 6 months follow-up
Shuai *et al.* 2012	Rat	EPCs	No malignant changes observed during 12 weeks follow-up
Togel *et al.* 2009	Rat	MSCs	No tumor formation observed during 4 weeks follow-up

hucMSCs - Human-umbilical cord-derived mesenchymal stem cells; MRPC - Mouse renal progenitor cells; HSPCs - Hematopoietic stem and progenitor cells; rKS56 - Proximal tubule S3 segment-derived renal progenitor-like cell line; iPS – Induced pluripotent stem cells; EPCs - Endothelial progenitor cells; MSCs - Mesenchymal Stem/Stromal Cells.
* Indirect evidence of tumorigenicity

Biodistribution of administered cells

The *in vivo* fate of genetically modified cells is another important factor related to the safety of a cell-based therapy. One of the main biodistribution-related concerns is the undesired migration of cells to non-target organs and tissues, which could cause a risk of developing a local inflammatory response, or neoplasm formation. It has been reported previously that the number of cells reaching desired tissues and organs can be very low (as minimal as 1%) due to the fact that the cells remain trapped within the lungs because of their size and high abundance of surface adhesion molecules, especially in the case of an intravenous administration route [64,65]. Therefore, monitoring cell distribution and migration in the body is of crucial importance when assessing safety aspects of cell-based therapies. The majority of the studies included in this systematic review (84 %; 41 studies) did evaluate at least some biodistribution-related outcomes, such as trafficking, homing, engraftment, differentiation, survival, or persistence of cells after administration. Of these studies, 20 focused only on cell engraftment and survival within kidneys, while 21 evaluated distribution in at least one or more additional organs, such as lung, liver, spleen, lymph nodes, stomach, intestine, muscle, brain, blood, bone marrow or even eye. The specific cell type, organ or tissue distribution, cell persistence and outcome are shown in Table 2.4. Due to large differences between studies in terms of cell type, administration route, number of cells, time point and method implemented to trace cell fate after administration, as well as kidney disease model applied, it is difficult to assess the biodistribution of genetically modified cells in kidney disease animal models. However, most studies included reported that cells could reach the kidney and survive for a variable period of time, ranging from 24 h up to one year. Also, all studies that evaluated distribution in organs other than the kidney mentioned cell accumulation in highly vascularized organs, such as spleen, liver and especially lungs. The study by Togel *et al.* [66] focused exclusively on monitoring distribution of MSCs in a mouse model of AKI, using bioluminescence imaging (BLI). They

showed that following intra-arterial injection in injured mice, cells tended to accumulate in areas corresponding to kidneys, while in healthy mice cells were distributed throughout the whole body, with eventual accumulation in the lungs. On the other hand, intravenous injection of cells led to a predominant and immediate cell accumulation in the lungs, both in healthy and AKI mice. This implies that the concern of causing respiratory and hemodynamic complications due to capillary clogging is paramount, especially in case of intravenous injections. Nonetheless, more than 50% of the biodistribution-relevant studies (Table 2.2) adopted the intravenous administration route for cell delivery. In addition, due to a low rate of retention and limited cell survival, large numbers of cells may be needed to achieve therapeutic effects, which would further increase the risk of pulmonary emboli. Besides this undesired effect of cell distribution on efficacy, engraftment of cells in non-target tissues might also be responsible for unwanted negative effects of cell therapies. For instance, different local environments could influence cell behavior and biological properties, thus potentially favoring harmful effects related to differentiation, especially if stem cells are used [67]. However, none of the included studies reported differentiation issues. Nevertheless, in light of the evidence presented, biodistribution-related effects should not be underestimated, but carefully and extensively evaluated, especially in terms of mode and site of administration, cell number and type, as well as methodologies employed for cell tracking and detection.

Table 2.4. Organ and tissue distribution of various cell types used in kidney disease animal models.

Author and Year	Cell type	Organ or tissue distribution	Cell tracking method	Outcomes
Baulier *et al.* 2014	afMSCs	Kidney, Lung, Spleen, Liver	Fluorescence (GFP)	Cells present in kidney 24h after transplantation. Inconsistent presence of cells in kidney 3 months after administration. Cells were undetectable in lungs, spleen and liver both after 24h and 3 months
Bian *et al.* 2014	MSCs	Kidney	Fluorescence (GFP)	At day 28 cells were present in injured kidneys (tubular epithelium, interstitial space, glomeruli and peritubular capillary plexus)
Chen *et al.* 2008	4E cell line	Kidney	Fluorescence (CM-Dil, α-SMA)	At 30 days after administration cells were present in injured kidney. Also at day 30 cells were positive for α-SMA and localized along the peritubular capillary area indicating endothelial transdifferentiation
Chen *et al.* 2011	hucMSCs	Kidney	Optical living body imaging	Cells were detectable in kidney 72h after administration
Eliopoulos *et al.* 2011	MSCs	Kidney	PCR (mouse Y chromosome-specific fragment amplification)	Cells were present after several days and up to 14 days
Ferenbach *et al.* 2010	Macrophages	Kidney, Liver, Spleen, Lung	Fluorescence (PKH26, cell membrane labeling)	24h after injection high cell densities were identified in the spleen and liver, low cell densities were present in kidney and lung
Franchi *et al.* 2014	MSCs	Whole body	BLI (D-luciferin) and Fluorescence (CM-Dil)	24h after transplantation cells were detected only in kidney. At day 3 after the administration cells localized mostly in glomeruli and persisted there for up to 4 weeks, with some cells expressing endothelial marker vWF
Furuichi *et al.* 2012	ADMSCs	Kidney, Lung, Brain, Spleen, Liver, Blood	Flow cytometry (GFP), IHC (anti-GFP)	After injection cells were mainly present in blood and lungs. Cells were present in all organs at day 4 post-injection, with decreased number in lungs. By day 7 after administration cells had almost disappeared from all organs
Gao *et al.* 2012	ADMSCs	Kidney	BLI (D-luciferase)	Cells highly present at day 1. Lower amount of cells found at day 14. At day 21 cells were undetectable
Gheisari *et al.* 2012	MSCs	Kidney, Lung	Flow cytometry (CellTracker Green), Fluorescence (GFP)	36h post-injection cells were present only in lungs but not in kidneys
Hagiwara *et al.* 2008	MSCs	Kidney	IHC (anti-human kallikrein)	Cells present in kidneys 48h after administration
Han *et al.* 2013	MRPC	Kidney	IHC (anti-GFP)	Cells present in kidney after 2, 4 and 7 days following administration
Harrison *et al.* 2013	HSPCs	Whole body	Fluorescence (EGFP), BLI (D-luciferin)	Up to 9 months after administration cells were found in spleen, liver (transdifferentiated into Kupffer cells), brain (transdifferentiated into microglial cells), kidney (transdifferentiated into inflammatory dendritic cells). Cells were still present 1 year after administration (shown by luciferase expression)
Huang *et al.* 2012	BM-MSCs	Kidney	Fluorescence (GFP)	Cells were detectable in kidney 72h after administration

Author and Year	Cell type	Organ or tissue distribution	Cell tracking method	Outcomes
Imberti *et al.* 2007	MSCs	Kidney	Fluorescence (PKH26, cell membrane labeling)	At day 4 post-injection cells were predominantly present in peritubular areas in kidneys
Kelley *et al.* 2013	Selected renal cells (SRCs)	Kidney	MRI (SPIO), Fluorescence (Rhodamine), NMR spectroscopy (CS-DM-Red (^{19}F))	Cells were present in kidneys at 24h post-injection. Diminished yet significant presence 7 days after administration
Kelly *et al.* 2010	NRK-52E	Kidney, Spleen, Lung	Fluorescence (GFP)	At day 7 after administration cells were more abundant in kidney compared to lung and spleen
Kelly *et al.* 2012	Renal tubule cells	Kidney, Lung, Spleen, Liver	Fluorescence (GFP), FISH (Y chromosome)	Cells present in kidneys 7 and 21 days post-injection. Cells were rarely detected in lungs, spleen and liver
Kelly *et al.* 2013	Renal tubule cells*	Kidneys, Lung, Liver, Spleen	FISH (Y chromosome), PCR (SRY male gene, SAA), Fluorescence (GFP)	Cells present in kidneys 14 weeks after the administration. At 14 weeks post injection only few cells were detected in lung, liver and spleen
Kinomura *et al.* 2008	rKS56	Kidney	IHC (Bluo-gal)	Following intra-arterial injection cells could not be detected in kidney. Upon subcapsular cell implantation cells were observed in the subcapsule and corticomedullary junction area at day 9 after transplantation
Lee *et al.* 2012	iPS	Kidney, Liver, Spleen, Stomach, Intestine, Muscle, Lung, Brain	IHC (anti-GFP)	48h after administration, engraftment of cells was highest in the kidneys, in the peritubular area, followed by the spleen and the liver
Li *et al.* 2006	BMDCs	Kidney	Fluorescence (EGFP)	At day 84 after administration cells were still present in kidneys (glomeruli)
Li *et al.* 2006(2)	BMDCs	Kidney	Fluorescence (EGFP, CD31, vWF)	At days 7, 14 and 28 after injection, cells were present in kidneys and co-localizing with CD31 and vWF indicating transdifferentiation into endothelial cells
Li *et al.* 2012	HSPCs (Lin$^-$)	Kidney, Lung, Liver, Spleen, Bone marrow, Blood	FISH (Y chromosome)	At days 7 and 28 post-injection only few cells were present in kidneys. Higher number of cells detected in spleen, liver, lungs, bone marrow and blood at day 1 post-injection. At day 3 cell number was decreased in all organs
Lin *et al.* 2003	HSPCs (Lin$^-$)	Kidney	FISH (Y chromosome), PCR (SRY male gene), IHC (X-Gal)	4 weeks after transplantation cells were present in kidneys. Cells presence was confirmed in renal tubules by IHC
Liu *et al.* 2011	MSCs	Kidney, Brain, Liver	ISH (Y chromosome)	At 7 day after administration cells were detected in kidney (outer medulla). The number of cells decreased by day 14 post-administration. No cells were found in brain and liver
Liu *et al.* 2013	BM-MSCs	Kidney	Fluorescence (BrdU, DNA labeling)	At day 7 after transplantation cells were present in kidney

Author and Year	Cell type	Organ or tissue distribution	Cell tracking method	Outcomes
Luo *et al.* 2014	BM-MSCs	Kidney, Lung, Spleen, peritoneal lymph nodes	Fluorescence (RFP)	24h after administration cells were found in the spleen, lymph nodes and the highest amount in lungs, but not in kidneys
Lv *et al.* 2014	MSCs	Kidney	IHC (anti-GFP)	Small amount of cells present around glomeruli and near blood vessels 24h after transplantation. Only few cells present in glomeruli at 8 weeks
Pacurari *et al.* 2013	EC	Kidney, Liver, Lung, Heart, Spleen	Fluorescence (GFP, anti-CD31), IHC (vWF)	At day 7 after administration small number of cells was detected in kidney and spleen, while no cells were found in liver, lung and heart
Prodromidi *et al.* 2006	BMDCs	Kidney	ISH (Y chromosome)	Cells were detected in kidneys (glomeruli and interstitium) after transplantation
Rampino *et al.* 2011	MSCs	Kidney, Lung, Spleen	IHC (anti-GFP)	At 24h after injection, cells were present in kidney (tubules, interstitium and glomeruli). At same time cells were occasionally present in lung and spleen. Cells also persisted in kidneys after 14 days (glomeruli)
Ruan *et al.* 2013	Fibroblasts (induced)	Kidney	Fluorescence (GFP)	At 8 weeks after transplantation cells were abundant in renal tubules, glomerular capillary loop and small arteries
Syres *et al.* 2009	BMDCs	Kidney, Eye, Brain, Muscle, Liver, Spleen, Heart	BLI (D-luciferin), Fluorescence (GFP), PCR (Ctns gene),	Cells were present at 2 and 4 months after injection. The number of cells increased over time in all organs
Togel *et al.* 2008	MSCs	Whole body	BLI (D-luciferin), PCR (luciferase gene expression)	10-15 min after injection cells were located mostly in kidneys in AKI animals, while in healthy animals they show whole body distribution, especially in lungs. At 24h post-injection cells are still present in kidneys and lungs of AKI animals. In liver no cells were detected. 7 days after injection cells were absent in lungs, kidneys, liver and spleen
Tsuda *et al.* 2010	FM-MSCs	Kidney, Liver, Lung, Spleen	IHC (anti-GFP)	24h after injection, cells were found in kidneys (glomeruli, proximal tubule, interstitial area), but also in lung, liver and spleen, with highest concentration in lungs, followed by liver, spleen and kidneys. At 7 days after injection, cells were not present in kidneys anymore, but still present in lung, liver and spleen
Tsuda *et al.* 2014	FM-MSCs	Kidney, Lung, Spleen, Liver	IHC (anti-GFP)	24h after administration cells were more abundant in lungs, but also present in liver, spleen and kidneys
Wise *et al.* 2014	MSCs	Kidney, Lung	BLI (D-luciferin)	1h post-administration cells were present in lungs. Subsequently cells migrated to kidneys and remain persistent up to 3 days after injection. At day 7 post-administration cells were undetectable
Yuzeng *et al.* 2014	MSCs	Kidney	Fluorescence (GFP)	At day 7 after transplantation cells were detectable in kidney (survival of approximately 75%)
Zhen-Qiang *et al.* 2012	BM-MSCs	Kidney	Fluorescence (Hoechst 33342 and anti-CK18)	At day 3 after transplantation cells were present in kidney renal tubules

Author and Year	Cell type	Organ or tissue distribution	Cell tracking method	Outcomes
Zhuo *et al.* 2013	MSCs	Whole body	BLI (D-luciferin)	Immediately after administration cells are localized in lungs. Cells disappeared completely at day 7 regardless the administration route

afMSCs - Amniotic fluid-derived mesenchymal stem cells; MSCs - Mesenchymal Stem/Stromal Cells; 4E - kidney-derived clonal cell line of MSC; hucMSCs - Human-umbilical cord-derived mesenchymal stem cells; ADMSCs - Adipose-derived mesenchymal stem cells; MRPC - Mouse renal progenitor cells; HSPCs - Hematopoietic stem and progenitor cells; BM-MSCs - Bone marrow-derived mesenchymal stem cells; BMDCs - Bone marrow-derived cells; SRCs - Selected renal cells; NRK-52E - Rat epithelial kidney cell line; rKS56 - Proximal tubule S3 segment-derived renal progenitor-like cell line; iPS – Induced pluripotent stem cells; EC – Endothelial cells; hRPTEC - Human renal proximal tubular epithelial cells; EPCs - Endothelial progenitor cells. GFP – Green fluorescent protein; α-SMA - α-Smooth muscle actin; BLI – Bioluminescence imaging; SPIO - Superparamagnetic iron oxide; IHC – Immunohistochemistry; MRI – Magnetic resonance imaging; NMR – Nuclear magnetic resonance; (F)ISH – (Fluorescence) *in situ* hybridization; SRY – Sex-determining region Y; vWF – Von Willebrand factor; RFP – Red fluorescent protein; CK-18 – Cytokeratin-18.
* Renal tubule cells - Mix of cells derived from proximal, ascending, collecting and distal tubules

Contamination with microorganisms and adventitious agents and reactivation of latent viruses

The risk of transmission of pathogens, such as bacteria, viruses, fungi or prions, is another important, yet not sufficiently investigated safety concern of cells and tissue xenografts and allografts [68,69]. This cannot be neglected, especially if the cells intended for use are of non-autologous origin and are genetically modified, cultured and expanded *in vitro*. Several studies have already described the undesired transmission of pathogens following cell transplantation [70,71]. Moreover, animal-derived products commonly used for the isolation, culture and propagation of cells, such as fetal calf or bovine serum, represent an additional risk of transfer of contaminants. In fact, due to its method of preparation, fetal bovine serum might be contaminated with mycoplasma, viruses, prions, or endotoxins, thus transmitting diseases. It also contains various biomolecules, such as non-human sialic acid, that could have xenoimmunogenic effects in hosts [39,72-75]. Considering all included studies, only Takahashi *et al.* [16] evaluated the effect of serum-free medium on lifespan-extended renal proximal tubular epithelial cells function in the bioartificial tubule device (BTD). In particular, cells modified with siRNA for $p16^{INK4a}$ were cultured either in 0.5% serum-containing renal cell growth medium or serum-free RELAR® medium, based on HFDM-1 synthetic medium for human fibroblasts supplemented with various recombinant hormones and growth factors for renal cell culture. The results obtained showed that both cells cultured in serum-containing and in serum-free medium presented almost the same growth rate in terms of population doublings and performed equally in BTD with respect to leakage of creatinine and reabsorption of water, glucose and sodium. In addition, the two types of cells exhibited very similar performance in AKI goats during the 26 h extracorporeal circulation with BTD regarding plasma activity or concentration of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatinine, uric acid, blood urea nitrogen, glucose, sodium, potassium, chloride, calcium and inorganic phosphate, as well as mRNA levels of IL-6, IL-10, IL-1β, TNF-α and IFN-γ in peripheral blood mononuclear cells (PBMC) following 8 h LPS challenge. In order to avoid ethical issues and controversial

procedures involved in harvesting serum from bovine fetuses, and to ensure animal and pathogen-free conditions of cell culture for safer tissue engineering and cell therapy applications, alternatives for fetal bovine serum are highly desired. Moreover, the possibility of reactivation of latent viruses, such as cytomegalovirus, herpes zoster or Epstein-Barr virus, with the production of infectious viral particles, is another point to be addressed [40]. However, we are aware that this aspect of safety is highly linked to the immunosuppressive therapy that might be required when allogeneic cells are applied, and that it can be more relevant in the clinical rather than the preclinical setting. None of the studies included in this review addressed this issue, but nevertheless it remains a valid point worth of consideration.

Animal survival

Of the studies included, 13 (27%) reported animal survival or mortality rate after cell administration (Table 2.2; outcome ''S''). However, in all cases the main focus and intention was not to evaluate possible negative or detrimental effects of genetically modified cells and administration route, but to determine the beneficial effect of cell therapy on the overall survival in a kidney disease setting. It would have been of great value if those studies had the appropriate control groups, as discussed earlier (Figure 2.2), which could give a fair indication of cell therapy effect on survival, without confounding variables such as kidney disease itself. In that regard, most of the included studies that reported any results on animal survival, had a healthy or sham operated control group, depending on the kidney disease model, a vehicle treated group such as PBS, and a group treated with genetically modified cells for therapeutic purposes. On rare occasions a group of animals with kidney disease treated with wild type cells, as a control for the group injected with genetically modified cells was included as well. Nonetheless, in the absence of a healthy or sham operated control group treated with genetically modified cells alone, the potential adverse effects of cell therapy on animal survival are difficult to evaluate.

Limitations and need for better-designed preclinical studies

As mentioned, the experimental design of evaluated studies was often inappropriate for evaluation of the risks associated with cell administration. Even if the main goal of the studies was to assess a therapeutic effect, we believe that additional test groups could have been included to evaluate safety. Notably, the most frequently missing animal group was a control group (eg. healthy or sham operated animals) in which the same cells were used as in the diseased group. In case of treatment with genetically modified cells, an additional control group of animals treated with wild type cells could also provide valuable information regarding the effect of cell therapy on overall animal well-being.

Besides, the studies included in this review are marked by high heterogeneity, especially in terms of the chosen cell type, cell number, route of administration, and, to some extent, cell source. In fact, cell number applied across all these studies was in the range of 10^3 – 10^8 (Table 2.2). This clearly indicates that the cell number required for optimal therapeutic results is not clear. Higher cell numbers might imply higher risk of developing certain side effects, such as lung

obstruction [65], but this also depends on the animal model and administration route chosen, as reported previously [66]. For that reason, a careful examination of cell biodistribution in animal experiments should be performed to determine the effectiveness and safety of a given cell type.

Another important issue is to ascertain the purity of a cell population that is intended for therapeutic use, even more so when cells underwent genetic modifications. With that in mind, the differentiation status of cells should be determined, by examining specific cell type markers and when possible cell specific functions, in order to avoid the undesired tumorigenic risks due to residual undifferentiated cells [76]. When poorly differentiated cells, or even stem cells, such as embryonic stem cells are used, the tumorigenic and teratogenic effects should be evaluated carefully [77].

The determination of cell culture purity is also necessary to evaluate the genetic changes that can occur in culture over time, or are a consequence of various genetic manipulations, such as transfection or transduction used to enhance cell function. Karyotype analysis should suffice to determine any significant chromosomal aberrations that might render the cell product unreliable for use, either from the functional point of view or based on cancer risk.

Moreover, when possible, cells should be tested in animal studies at higher passage numbers, usually beyond the routine use, to ensure the safety related to tumorigenicity and immunogenicity, as already suggested [78]. When using allogeneic or xenogeneic cells, it is extremely important to characterize cells for their immunogenic potential by assessing expression of immune related molecules and antigens, but also by evaluating cells persisting at the site of transplantation to check for cell survival, inflammatory cell infiltration, T cell activation, and cytokine and antibody levels, as indicators of graft rejection and immune system activation [79-82]. In case of genetically modified cells, major attention should be given to the expression of transgenes, used either for improving cell function or for cell tracking, since the particular gene products can be immunogenic as was shown for GFP [83].

Finally, manufacturing processes of cells and cell-based therapies do not provide any viral removal or inactivation and sterilization. Considering that most cells are cultured and expanded in fetal bovine serum-containing growth media, continuous testing for microorganisms should be performed to maintain microbial safety of cell based therapeutic products [39]. In addition to ensuring microbial safety, use of serum-free culture media could help standardize the cell expansion and manufacturing procedures that are subject to serum batch-to-batch variations [72,73,84-86].

Even though there are several official guidelines [35,36,40,78] for safety assessment of cell therapies and medicinal products based on cells and tissues, poor preclinical practice makes it difficult for regulatory agencies to establish new or improve currently existing guidelines and recommendations for safety evaluation. Overall, better design and execution of preclinical studies could drastically improve the safety evaluation of genetically modified cell therapy in kidney disease.

CONCLUSIONS

We identified 49 studies describing the use and efficacy of genetically modified cells for the treatment of AKI and CKD in animals. However, only 3 of these studies (6%) assessed the safety aspects of such therapies in a sufficient manner. Based on the current findings and observations it seems that various cell types employed for kidney disease treatment do not carry significant risk factors and side effects, such as tumorigenic and teratogenic effects. However, given that most of the studies did not have the optimal design, with 69% designed poorly or inappropriately, and that they were highly heterogeneous with respect to animal species, disease model and cell therapy, it is rather challenging to get a general overview on safety aspects of cell-based therapies. Hence, we encourage further research with well-designed preclinical studies according to the guidelines and recommendations, in order to better define adverse events potentially involved in kidney cell therapy. The most relevant safety-related outcomes are those regarding the purity, biodistribution and immunotoxic effects, as well as the tumorigenic potential related to genetic modifications, genomic instability and differentiation level of cells.

ACKNOWLEDGMENTS

This research was supported by the Marie Curie ITN project BIOART (grant no. 316690, EU-FP7-PEOPLE-ITN-2012) and ZonMW (project code 114024113).

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SUPPLEMENTARY INFORMATION

Table 2.S1. Full search strategy for PubMed and EMBASE

PubMed	Cell and tissue based therapy	Cell- and Tissue-Based Therapy [Mesh] OR Cell Therapy [tiab] OR Cell Therapies [tiab] OR Cell Therapy [ot] OR Cell Therapies [ot] OR Cellular Therapy [tiab] OR Cellular Therapies [tiab] OR Cellular Therapy [ot] OR Cellular Therapies [ot] OR Cell Transplantation [Mesh] OR Cell Transplantation [tiab] OR Cells Transplantation [tiab] OR Cell Transplantation [ot] OR Cells Transplantation [ot] OR Transplanted Cells [tiab] OR Transplanted Cell [tiab] OR Transplanted Cells [ot] OR ((Cell-based [tiab] OR Cell based [tiab] OR Cell-mediated [tiab] OR Cell mediated [tiab]) AND (Therapy [tiab] OR Therapies [tiab] OR Treatment [tiab])) OR Tissue Engineering [Mesh] OR Tissue Engineer* [tiab] OR Tissue Engineer* [ot] OR Regenerative medicine [Mesh] OR Regenerative medicine [tiab] OR Regenerative medicine [ot] OR ((transformed [tiab] OR immortaliz* [tiab] OR immortalis* [tiab] OR continuous [tiab] OR permanent [tiab]) AND (Cell line [tiab] OR Cell lines [tiab])) OR immortaliz* [ot] OR immortalis* [ot] NOT (vaccine [tiab] or vaccine [ot] or characterization [tiab] or tolerance [tiab]).
	Kidney disease	(kidney diseases [MeSH Terms] OR acute kidney injury [MeSH Terms] OR Kidney Failure, Chronic [Mesh] OR Renal Insufficiency[Mesh] OR kidney disease [Tiab] OR kidney injury [tiab] Or kidney ischemia [tiab] or kidney diseases [Tiab] OR kidney failure[tiab] OR kidney fibrosis [tiab] OR renal disease[tiab] OR renal diseases [tiab] OR renal injury[tiab] OR renal ischemia[Tiab] OR renal failure [tiab] OR renal insufficiency [tiab] OR renal fibrosis[tiab] OR kidney disease [ot] OR kidney injury [ot] OR kidney ischemia [ot] OR kidney diseases [ot] OR kidney failure[ot] OR kidney fibrosis [ot] OR renal disease [ot] OR renal diseases [ot] OR renal injury[ot] OR renal ischemia[ot] OR renal failure [ot] OR renal insufficiency [ot] OR renal fibrosis [ot] OR Chronic Kidney Disease [tiab] OR Chronic Kidney Disease [ot]).
	Animals	Laboratory animal search filter [87]
Embase	Cell and tissue based therapy	exp cell therapy/ or exp cell transplantation/or (Cell Therapy or Cell Therapies or Cell Transplantation or Cells Transplantation or Transplanted Cells or Transplanted Cell).ti,ab,kw. or ((Cell-based or Cell based or Cell-mediated or Cell mediated or Cellular) and (Therapy or Therapies)).ti,ab,kw. or ((Cell-based or Cell based or Cell-mediated or Cell mediated or Cellular) and (Treatment)).ti,ab. or exp tissue engineering/ or exp regenerative medicine/ or exp immortalized cell line/ or exp transformed cell line/ or (Tissue Engineer* or Regenerative medicine).ti,ab,kw. or ((transformed or immortaliz* or immortalis* or continuous or permanent) and (Cell line or Cell lines)).ti,ab,kw. NOT (vaccine or characterization or tolerance).ti,ab,kw .
	Kidney disease	exp kidney injury/ OR exp artificial kidney/ OR exp kidney disease/ OR exp kidney failure/ OR (renal disease OR renal diseases OR renal ischemia OR renal injury OR renal insufficiency OR renal fibrosis OR renal failure OR kidney injury OR kidney disease OR kidney diseases OR kidney ischemia OR kidney fibrosis OR kidney failure).ti,ab,kw.
	Animals	Laboratory animal search filter [88].

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International Journal of Molecular Sciences. 2017; 18(12), 2531
doi:10.3390/ijms18122531

CHAPTER III

ROLE OF VITAMIN D IN MAINTAINING RENAL EPITHELIAL BARRIER FUNCTION IN UREMIC CONDITIONS

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ABSTRACT

As current kidney replacement therapies are not efficient enough for end-stage renal disease (ESRD) treatment, a bioartificial kidney (BAK) device, based on conditionally immortalized human proximal tubule epithelial cells (ciPTEC), could represent an attractive solution. The active transport activity of such a system was recently demonstrated. In addition, endocrine functions of the cells, such as vitamin D activation, are relevant. The organic anion transporter 1 (OAT1) overexpressing ciPTEC line presented 1α-hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1) and vitamin D receptor (VDR), responsible for vitamin D activation, degradation and function, respectively. The ability to produce and secrete 1α,25-dihydroxy-vitamin D_3, was shown after incubation with the precursor, 25-hydroxy-vitamin D_3. The beneficial effect of vitamin D on cell function and behavior in uremic conditions was studied in the presence of an anionic uremic toxins mixture. Vitamin D could restore cell viability, and inflammatory and oxidative status, as shown by cell metabolic activity, interleukin-6 (IL-6) levels and reactive oxygen species (ROS) production, respectively. Finally, vitamin D restored transepithelial barrier function, as evidenced by decreased inulin-FITC leakage in biofunctionalized hollow fiber membranes (HFM) carrying ciPTEC-OAT1. In conclusion, the protective effects of vitamin D in uremic conditions and proven ciPTEC-OAT1 endocrine function encourage the use of these cells for BAK application.

Keywords: bioartificial kidney; conditionally immortalized proximal tubule cells; chronic kidney disease; end-stage renal disease; vitamin D; uremic toxins; epithelial barrier

INTRODUCTION

It has been reported that chronic kidney disease (CKD), defined as the sustained presence of a decreased glomerular filtration rate (GFR) with or without increased albumin excretion, has a rather high global prevalence, estimated to be between 11% and 13% [1]. The progressive loss of kidney function will ultimately lead to a permanent state of end-stage renal disease (ESRD). Kidney failure is accompanied by a noticeable accumulation of a variety of endogenous uremic metabolites that are not efficiently cleared by the kidneys, leading to a broad range of pathologies, mostly cardiovascular disease and bone disorders, with reduced quality of life, as well as significantly increased mortality [2-4]. Although kidney transplantation is the treatment of choice for most patients with ESRD, patients who are older or have significant comorbidity are not eligible for transplantation. Moreover, due to a shortage of donor organs, dialysis therapy is frequently required during the waiting time for transplantation. However, both hemodialysis and peritoneal dialysis are inefficient techniques for the removal of waste products, especially larger and protein-bound uremic toxins [5]. Moreover, hemodialysis decreases the amount of vitamin D in serum [6].

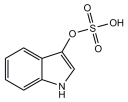
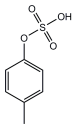
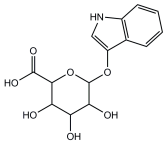
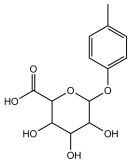
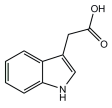
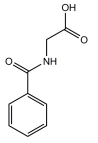
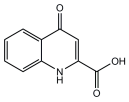
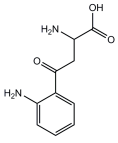
The kidney also has intrinsic endocrine activity, producing hormones and immunomodulatory molecules. One of the essential hormones is 1α,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$; calcitriol), the most active form of vitamin D, which is often deficient in CKD and ESRD

populations, giving rise to severe comorbidities [7, 8]. Normally, 25-hydroxy-vitamin D_3 (25(OH)D_3), mostly bound to vitamin D binding protein (VDBP), is taken up in proximal tubular epithelial cells (PTEC) by the multiligand binding receptor megalin (also known as low-density lipoprotein-related protein 2; LRP2) from the glomerular ultrafiltrate and subsequently converted by 1α-hydroxylase (CYP27B1) to 1,25$(OH)_2D_3$. 1,25$(OH)_2D_3$ has both autocrine and endocrine functions [8, 9], and by binding to the intracellular vitamin D receptor (VDR) it is able to control the expression of genes involved in the regulation of skeletal health, but it can also have a range of other functions with effects on the cardiovascular and immune systems [10-12]. The activity of 24-hydroxylase (CYP24A1) is responsible for maintaining vitamin D homeostasis when present in high serum concentrations, as it catalyzes 1,25$(OH)_2D_3$ oxidation to inactive metabolites in PTEC that can be excreted in the urine [13, 14]. It has been described that CKD patients have a progressive reduction in 1,25$(OH)_2D_3$ serum levels due to a lower glomerular filtration rate, limited 1α-hydroxylase activity, and lower megalin content [8, 15], but an increase in CYP24A1 levels has also been reported [16]. The vitamin D deficiency in these patients is directly associated with mineral bone disorders, hyperphosphatemia and cardiovascular disease, which leads to accelerated disease progression and eventually death [17]. Moreover, vitamin D deficiency has been associated with epithelial barrier dysfunction and intestinal permeability in inflammatory bowel diseases (IBD), mostly due to the alteration of gut microbiome composition [18, 19].

Because of the shortcomings of current dialysis techniques, novel or improved therapies that can actively secrete waste molecules and replace essential metabolic kidney functions are being sought intensively. One of the most promising solutions is a bioartificial kidney device (BAK), composed of PTEC cultured on hollow fiber membranes (HFM) to mimic proximal tubule physiology [20]. Since the first time BAK was introduced as a possible replacement kidney therapy, many studies have focused on the development and characterization of such devices [21-25], including human clinical trials [26], with the main limitations related to the choice of cells that would be safe and efficient enough, and readily available at the same time. A unique cell type created by our group is represented by urine-derived conditionally immortalized PTEC (ciPTEC) [27, 28] equipped with the organic anion transporter 1 (OAT1) [29]. This transport protein is responsible for the uptake of many anionic waste products in kidney proximal tubule, as a first step in their renal excretion. We recently described the successful culture of ciPTEC-OAT1 on HFM and active transport of uremic toxins as active BAK component [30-32].

The present study was designed to characterize ciPTEC-OAT1 for the expression of genes responsible for vitamin D metabolism and function, as well as its activation to the most potent form, 1,25$(OH)_2D_3$. In addition, we evaluated the effect of a specific mixture of eight anionic uremic toxins (Table 3.1), mimicking uremic conditions of CKD and ESRD, on vitamin D activation and function. In particular, we focused on the beneficial effects of vitamin D on cell viability, oxidative stress, inflammation and epithelial monolayer barrier function of ciPTEC-OAT1 cultured on biofunctionalized polyethersulfone HFM. The ability of ciPTEC-OAT1 to produce vitamin D, exerting protective effects on cells, could greatly improve both BAK function and application as a treatment modality.

Table 3.1. Concentrations of anionic uremic toxins in healthy individuals, uremic patients, and as applied in the present study. Concentrations used are adapted from EUToX Uremic Solutes Database (http://uremic-toxins.org/DataBase.html) and Jansen *et al.* [33].

Compound	Normal conc. (μM) (mean±SD)	Uremic conc. (μM) (mean±SD)	1x UT mix (μM)	Structure
Indoxyl sulfate	2.3±18.8	173.5±121.9	100	
p-cresyl sulfate	10.1±12.2	122.2±90.3	500	
Indoxyl-β-glucuronide	3.1±1.3	9.4±9.4	10	
p-cresyl glucuronide	0.3±0.2	30.1±6.7	40	
Indol-3-acetic acid	2.9±1.7	11.4±2.3	3	
Hippuric acid	16.7±11.2	608.4±362.8	300	
Kynurenic acid	0.03±0.01	0.8±0.4	3	
L-kynurenine	1.9	3.3±0.9	5	

MATERIALS AND METHODS

Reagents

All reagents (including all but two of the uremic toxins) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. The uremic toxins p-cresyl sulfate (pCS) and p-cresyl glucuronide (pCG) were synthesized by the Institute for Molecules and Materials, Radboud University, Nijmegen, The Netherlands, as described [32]. Ketoconazole, 1α,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) and 25-hydroxy-vitamin D_3 ($25(OH)D_3$) were purchased form Enzo Life Sciences (Raamsdonksveer, The Netherlands). MicroPES type TF10 hollow fiber capillary membranes (wall thickness 100 µm, inner diameter 300 µm, max pore size 0.5 µm) were purchased from Membrana GmbH (Wuppertal, Germany). Cell culture plates were obtained from Greiner Bio-One (Monroe, NC, USA).

Cell Culture of ciPTEC-OAT1

The ciPTEC-OAT1 cell line was cultured as reported previously [29]. Briefly, cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1 DMEM/F-12) (Gibco, Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium, 35 ng/mL hydrocortisone, 10 ng/mL epidermal growth factor and 40 pg/mL tri-iodothyronine to form a complete culture medium, without addition of antibiotics and up to a maximum of 60 passages. Cells were cultured at 33 °C and 5% (*v*/*v*) CO_2 to allow proliferation and prior to the experiments seeded at a density of 55,000 cell/cm^2. Subsequently, cells were grown for one day at 33 °C, 5% (*v*/*v*) CO_2 to allow adhesion, then cultured for seven days at 37 °C, 5% (*v*/*v*) CO_2 for differentiation and maturation, refreshing the medium every other day.

ciPTEC-OAT1 Exposure to Uremic Toxins Mixture

In order to replicate the uremic conditions present in kidney patients, a specific mixture of eight known anionic uremic toxins (Table 3.1), predominantly derived from endogenous metabolism pathways and food digestion in the gut [33], and corresponding approximately to the concentrations found in patients (1×), or higher (2.5×, 5× and 10×) (Table 3.1), was used in the present study. It was prepared as a 100× concentrated mixture in a serum-free medium and subsequently diluted to desired concentrations.

Cell Viability Assay

Cell viability was measured using PrestoBlue® cell viability reagent (Life Technologies). After seven days of maturation, cells were exposed to increasing concentrations of $1,25(OH)_2D_3$ (100 nM, 500 nM, 1 µM), anionic UT mix (1-, 2.5-, 5-, or 10-times concentrated) and a combination of $1,25(OH)_2D_3$ and UT mix in the previously mentioned concentrations. Following 24 h incubation at 37 °C, 5% (*v*/*v*) CO_2, ciPTEC were rinsed once with Hank's Balanced Salt Solution (HBSS; Gibco, Life Technologies) and incubated with PrestoBlue® cell viability reagent (diluted

1:10 in complete culture medium), in the dark. After 1 h incubation at 37 °C, 5% (*v*/*v*) CO_2, the fluorescence was measured using a fluorescent microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific, Vantaa, Finland), at excitation wavelength of 530 nm and emission wavelength of 590 nm. Data were corrected for the background, normalized to untreated cells, and presented as relative cell viability.

RNA Extraction, cDNA Synthesis, and Real-Time PCR

Total RNA from ciPTEC-OAT1 exposed to $1,25(OH)_2D_3$ (100 nM and 1 μM) and UT mix (1× and 2.5×) for 24 h, was isolated using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions and quantified using the NanoDrop® ND-1000 spectrophotometer. Reverse transcription of RNA to complementary DNA (cDNA) was performed using the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA) following manufacturer's instructions. Subsequently, Real-Time PCR was performed using the iQ SYBR® Green Supermix (Bio-Rad Laboratories) as indicated in manufacturer's protocol and by means of CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories). The data were analyzed using Bio-Rad CFX Manager™ Software version 3.1 (Bio-Rad Laboratories) and expressed as relative gene expression, using untreated cells as the reference sample. HPRT1 was used as a housekeeping gene for normalization. Specific sense and anti-sense primers for HPRT1 (forward: ACATCTGGAGTCCTATTGACATCG; reverse: CCGCCCAAAGGGAACTGATAG), VDR (forward: CTGACCCTGGAGACTTTGAC; reverse: TTCCTCTGCACTTCCTCATC), 1α-hydroxylase, (forward: GGCAGAGTCTGAATTGCAAAT; reverse: CCGGGTCTTGGGTCTAACTG), CYP24A1 (forward: GGCCTCTTTCATCACAGAGCT; reverse: GCCTATCGCGACTACCGCAA), ZO-1 (forward: ATGGTGTCCTACCTAATTCAACTCAT; reverse: GCCAGCTACAAATATTCCAACATCA) and claudin 2 (forward: ACCTGCTACCGCCACTCTGT; reverse: CTCCCTGGCCTGCATTATCTC) were synthesized by Biolegio (Nijmegen, The Netherlands).

Agarose Gel Electrophoresis

Real-time PCR products of the VDR, 1α-hydroxylase and CYP24A1 genes were detected by agarose gel electrophoresis. An 1.5% agarose gel was prepared in Tris-Borate-EDTA (TBE) buffer, including the SYBR™ Safe DNA gel stain (1:10,000) (Invitrogen, Carlsbad, CA, USA) for visualization of cDNA fragments. Loading buffer (30% glycerol, 0.25% bromophenol blue) was added 1:6 to the PCR product samples prior to loading them in the agarose gel. The 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used to determine the size of the fragments. Electrophoresis was conducted at 24 W and 100 V using Bio-Rad PowerPac™ HC power supply (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, the fragments were visualized using ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) and data analyzed by means of Image Lab software (version 5.2, Bio-Rad Laboratories).

Quantification of 1α,25-Dihydroxy-Vitamin D_3

Matured ciPTEC-OAT1 were exposed to 25(OH)D_3 100 nM, ketoconazole 10 μM and the co-treatment, as well as 1× UT mix alone and in the presence of 25(OH)D_3 100 nM, ketoconazole 10 μM or both of them. Ketoconazole was always added 2 h prior to the treatment with 25(OH)D_3 and UT mix. After 24 h incubation, cell culture supernatants were collected, centrifuged for 10 min at 240× *g*, 4 °C, and stored at −80 °C. 1,25$(OH)_2D_3$ was quantified after immuno-extraction using a competitive RIA (IDS AA-54F1; IDS Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany).

Enzyme-Linked Immunosorbent Assay (ELISA)

The production of IL-6 and TNF-α was measured using the Enzyme-Linked Immunosorbent Assay (ELISA). Cell culture supernatants were collected after 24-h treatments with 1,25$(OH)_2D_3$ (100 nM, 500 nM, 1 μM), UT mix (1 and 2.5×), a combination of 1,25$(OH)_2D_3$ and UT mix in the previously mentioned concentrations, as well as LPS (*Escherichia coli* 0127:B8) 10 μg/mL alone or as a co-treatment with increasing concentrations of 1,25$(OH)_2D_3$. Afterwards, cell culture supernatants were centrifuged for 10 min, 240× *g*, 4 °C, and stored at −20 °C. DuoSet® ELISA Development Systems kits (IL-6 #DY206, TNF-α #DY210; R&D Systems, Abingdon, UK) were used to quantify the cytokines levels in complete cell culture medium supernatants following manufacturer's instructions. The optical density was determined using the iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA) set to 450 nm. Each sample was measured in duplicates and quantification was done using Microplate Manager software (version 6.0, Bio-Rad Laboratories), generating a four parameter logistic (4-PL) curve-fit.

Intracellular Reactive Oxygen Species (ROS) Detection

Intracellular ROS generation was measured by means of cell permeant fluorogenic substrate 2′,7′-dichlorofluorescein diacetate (H_2DCFDA). Briefly, cells were washed once with HBSS, immediately loaded with H_2DCFDA (50 μM in serum-free medium) and incubated at 37 °C, 5% (*v*/*v*) CO_2, in the dark for 45 min. Afterwards, cells were washed with a complete culture medium and exposed to various concentrations of 1,25$(OH)_2D_3$ (500 nM and 1 μM) and 5× UT mix for 2 h at 37 °C, 5% (*v*/*v*) CO_2, in the dark. H_2O_2 (100 μM and 200 μM) was used as a positive control. Following the incubation, cells were washed twice with HBSS and lysed using 0.1 M NaOH for 10 min. Finally, fluorescence was measured at an excitation wavelength of 492 nm and emission wavelength of 518 nm, using the Fluoroskan Ascent FL microplate reader. Measured fluorescence values were corrected for the fluorescence of the blank sample (non-stained lysed cells) and used to calculate relative ROS production, using untreated cells as a reference.

CiPTEC-OAT1 Epithelial Monolayer Integrity

To investigate the effect of vitamin D on epithelial monolayer barrier function in uremic conditions, ciPTEC-OAT1 were cultured on L-3,4-dihydroxyphenylalanine (L-DOPA; 2 mg/mL) and collagen IV (25 μg/mL) coated HFM, mounted on a tailor-made flow chamber as described

previously [30, 32]. HFM with untreated mature ciPTEC-OAT1 monolayers and those exposed to 1,25$(OH)_2D_3$ (1 μM), 2.5× UT mix or a combination of both, were perfused with inulin-FITC (0.1 mg/mL) in Krebs–Henseleit buffer supplemented with 10 mM HEPES, pH 7.4, for 10 min. Next, aliquots from the apical compartment were collected and used to measure fluorescence by means of the Fluoroskan Ascent FL microplate reader, at excitation wavelength of 492 nm and emission wavelength of 518 nm. Background values were subtracted and normalized arbitrary fluorescence unit (AFU) data were converted and plotted as nmol·min^{-1}·cm^{-2}, as described previously [32]. From each single replicate (fiber), three different regions, with an area of 0.157 cm^2, were analyzed.

Immunocytochemistry

To assess the expression of tight junction protein ZO-1, ciPTEC-OAT1 cultured on double-coated HFM were fixed with 4% (*w*/*v*) paraformaldehyde dissolved in PHEM buffer (120 mM PIPES, 50 mM HEPES, 4 mM $MgCl_2$, 20 mM EGTA) for 15 min. After washing the samples with HBSS, block solution (2% (*v*/*v*) FCS, 2% (*w*/*v*) bovine serum albumin (BSA), 0.1% (*v*/*v*) Tween20 in HBSS) was added. The primary antibody, rabbit anti-human ZO-1 (Invitrogen, Carlsbad, CA, USA), was diluted in blocking buffer (1:200) and incubated overnight at 4 °C. Following three washing steps with HBSS, the secondary antibody, goat anti-rabbit IgG Alexa 568 (Life Technologies, Eugene, OR, USA) was added in a concentration of 1:200 and incubated for 1 h at room temperature. Finally, ProLong™ Gold antifade reagent containing DAPI (Life Technologies, Eugene, OR, USA) was used for nuclear staining, and to mount the fibers containing cells on the Willco glass bottom dishes (WillCo Wells B.V., Amsterdam, The Netherlands). Cells were imaged using confocal microscope (Leica TCS SP8 X, Leica Microsystems CMS GmbH, Wetzlar, Germany) and analyzed using Leica Application Suite X software (Leica Microsystems CMS GmbH).

Data Analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. A *p*-value < 0.05 was considered significant. Datasets were assessed for normality and equal variances assumptions prior to one-way ANOVA, using Kolmogorov–Smirnov and Bartlett's tests, respectively. Even though some datasets did not meet one of the assumptions, due to a limited number of measurements, the expected effect on the Type I error in one-way ANOVA is minimal. Software used for statistical analysis was GraphPad Prism (version 6.07; GraphPad software, La Jolla, CA, USA). In most experiments at least three independent experiments were performed in duplicates, unless otherwise stated. The exact sample size for each experiment is indicated in the corresponding figure legend.

RESULTS

Expression of Vitamin D Metabolism and Function-Related Genes in ciPTEC-OAT1

In ciPTEC-OAT1, the expression of genes involved in vitamin D metabolism, activation and degradation, 1α-hydroxylase and CYP24A1, respectively, and of VDR was confirmed by real-time PCR (Figure 3.1a). Agarose gel electrophoresis confirmed the specificity of the primers as the size of the PCR products corresponded to the expected amplicon length (Figure 3.S1). The housekeeping gene used for normalization was HPRT1, whose expression levels did not change upon various stimulations (Figure 3.S2). Moreover, vitamin D's effect on the expression of these genes was examined after 24 h exposure to either 100 nM or 1 μM of $1,25(OH)_2D_3$. While no significant impact on VDR expression was found, the gene expression of the two enzymes was significantly affected. In fact, an almost 50% reduction in 1α-hydroxylase expression was observed after $1,25(OH)_2D_3$ treatment when used at 1 μM, and a more than 1000-fold increase in CYP24A1 expression, regardless of the vitamin D concentration used (Figure 3.1b). No significant changes in gene expression were observed in the presence of a uremic toxins mixture (UT mix) at 1× or 2.5× concentrations (Figure 3.1c), which was used to mimic the conditions of kidney patients.

Conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ by ciPTEC-OAT1

To assess whether ciPTEC-OAT1 are able to produce the most active form of vitamin D, $1,25(OH)_2D_3$, cells were exposed to 100 nM $25(OH)D_3$ for 24 h, in the presence or absence of 1α-hydroxylase inhibitor—ketoconazole (10 μM). Measured levels of $1,25(OH)_2D_3$ confirmed that ciPTEC-OAT1 did produce the active form of vitamin D and the conversion was sensitive to inhibition by ketoconazole (Figure 3.2a). Uremic conditions (1× UT mix) did not influence the vitamin D activation (Figure 3.2b).

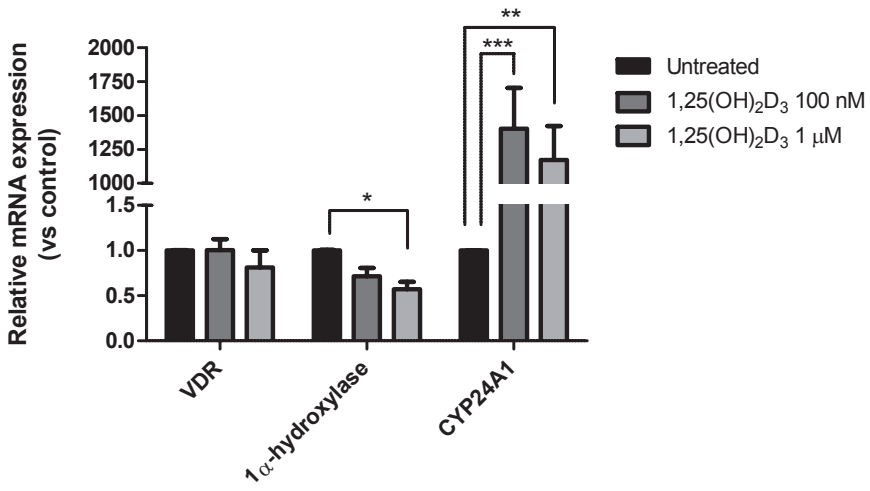
Protective Effect of $1,25(OH)_2D_3$ on Anionic Uremic Toxin Mix Induced Cell Toxicity

To further examine the effect of $1,25(OH)_2D_3$ on ciPTEC-OAT1 viability in normal and uremic conditions, cells were exposed to several concentrations of $1,25(OH)_2D_3$ in the absence or presence of increasing concentrations of UT mix. As shown in Figure 3.3a, the active form of vitamin D alone did not compromise cell viability. However, anionic uremic toxins did reduce cell viability after 24 h incubation, by approximately 10%, 25%, and 62% for 2.5×, 5×, and 10× concentrated mixtures, respectively (Figure 3.3b). Co-incubation of $1,25(OH)_2D_3$ with UT mix could mitigate the decrease in cell viability, especially when toxicity was induced by higher concentrations of UT mix (5× and 10×; Figure 3.3c).

(a)

	Ct (Mean ± SEM)
VDR	30.8 ± 0.11
1 α-hydroxylase	28.5 ± 0.15
CYP24A1	35.0 ± 0.08
HPRT1	24.3 ± 0.16

(b)



(c)

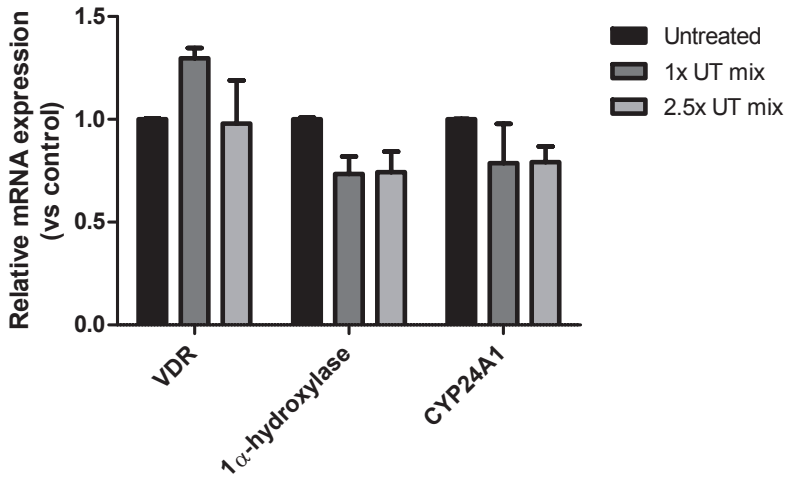


Figure 3.1. Expression of genes related to vitamin D function and metabolism in ciPTEC-OAT1. (**a**) Cycle threshold (Ct) values (expressed as mean ± SEM) reflecting expression levels in basal conditions of vitamin D receptor (VDR), 1 α-hydroxylase and CYP24A1. Relative mRNA expression of three genes in ciPTEC-OAT1 after 24 h exposure to (**b**) 100 nM and 1 μM of $1,25(OH)_2D_3$ or (**c**) 1× and 2.5× UT mix, compared to control (untreated ciPTEC-OAT1). Three independent experiments were performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA, Dunnett's multiple comparison test).

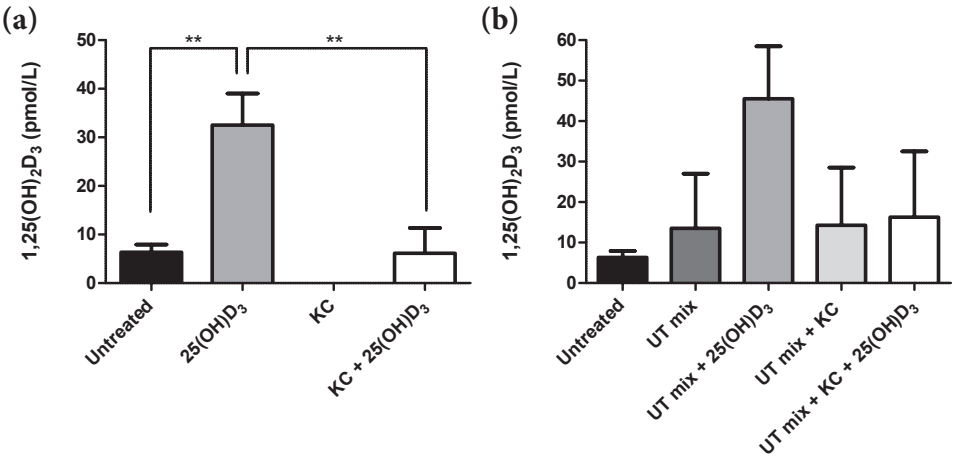


Figure 3.2. Conversion of vitamin D precursor to biologically active $1,25(OH)_2D_3$ by ciPTEC-OAT1. (**a**) Release of $1,25(OH)_2D_3$ in cell culture supernatant after 24 h incubation with inactive $25(OH)D_3$ (100 nM), in absence or presence of 1 α-hydroxylase inhibitor, ketoconazole (KC; 10 μM). (**b**) Release of $1,25(OH)_2D_3$ after 24 h incubation with $25(OH)D_3$ (100 nM), in absence or presence of ketoconazole (10 μM) and 1× UT mix. Concentration expressed as pmol/L (mean ± SEM). Three independent experiments were performed. ** $p < 0.01$ (One-way ANOVA followed by Dunnett's multiple comparison test, using as a control either untreated sample or $25(OH)D_3$ treated sample, as indicated).

Protective Effect of $1,25(OH)_2D_3$ on Anionic Uremic Toxin Mix Induced Oxidative Stress

To evaluate ciPTEC-OAT1 susceptibility to oxidative stress in uremic conditions and the anti-oxidative effect of $1,25(OH)_2D_3$, intracellular reactive oxygen species (ROS) generation was measured. Cells were exposed to 5× UT mix, $1,25(OH)_2D_3$ (500 nM or 1 μM) or a combination of UT mix and $1,25(OH)_2D_3$ for 2 h. UT mix induced a 1.5-fold increase in ROS production, which was attenuated significantly when adding vitamin D as a co-treatment, regardless of concentration (Figure 3.4). Also, the positive control H_2O_2 (200 μM) significantly enhanced ROS generation (Figure 3.4). Using 10× UT mix, similar effects of vitamin D on intracellular ROS levels were observed (Figure 3.S3a).

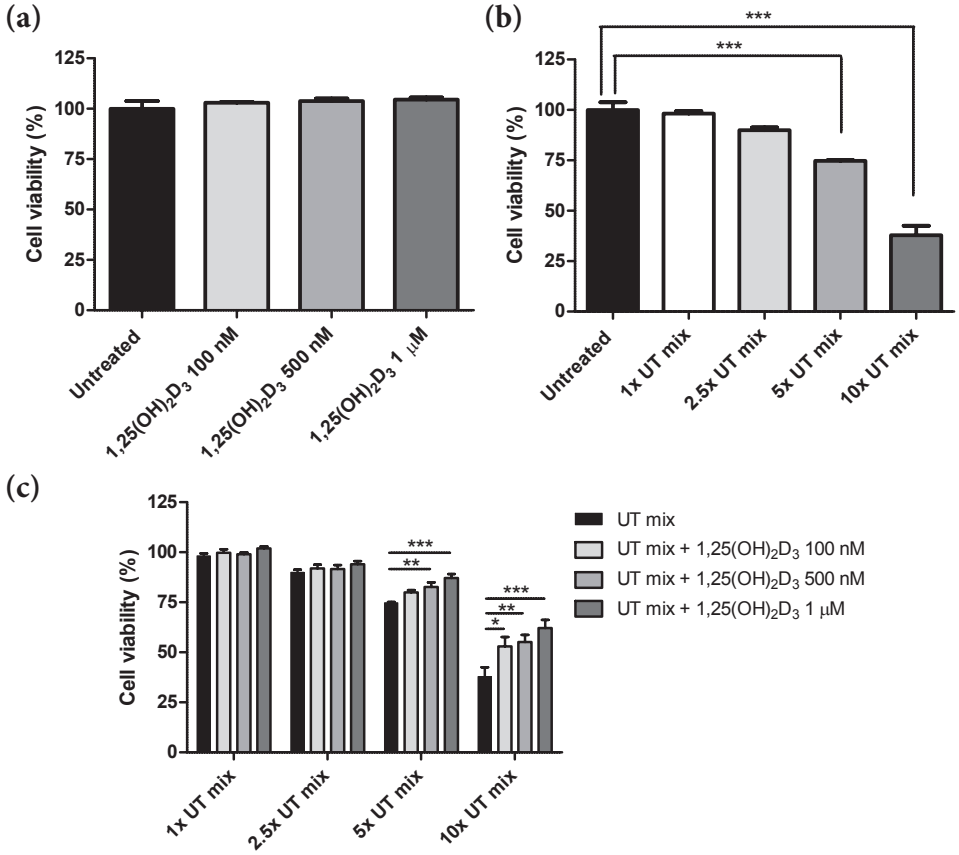


Figure 3.3. Effect of vitamin D on ciPTEC-OAT1 viability in uremic conditions. CiPTEC-OAT1 viability (relative to untreated cells) following 24 h exposure to (**a**) $1,25(OH)_2D_3$ alone (100 nM, 500 nM and 1 μM), (**b**) increasing concentrations of UT mix (1×, 2.5×, 5×, and 10×), and (**c**) combination of $1,25(OH)_2D_3$ and UT mix at all mentioned concentrations. Four independent experiments were performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA, Dunnett's multiple comparison test).

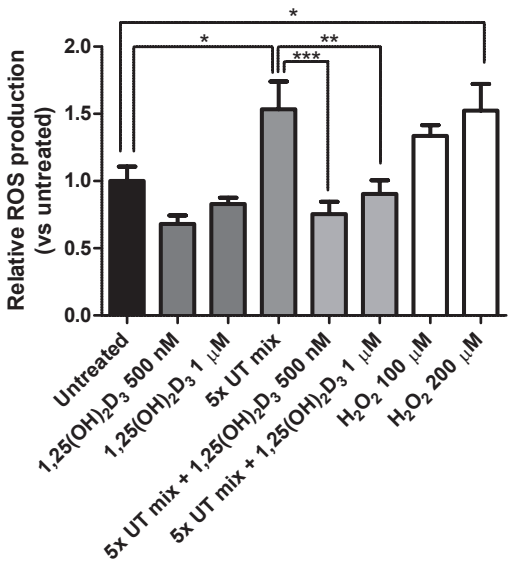


Figure 3.4. Vitamin D effect on intracellular ROS production in ciPTEC-OAT1. Relative ROS production in ciPTEC-OAT1 after 2 h exposure to $1,25(OH)_2D_3$ (500 nM and 1 μM), 5× UT mix, combination of the two at previous concentrations and H_2O_2 (100 μM and 200 μM). Three independent experiments were performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA followed by Dunnett's multiple comparison test, using as a control either untreated sample or 5× UT mix, as indicated).

Anti-Inflammatory Effect of $1,25(OH)_2D_3$ in Inflammatory and Uremic Conditions in ciPTEC-OAT1

Interleukin-6 (IL-6) levels in cell culture supernatant were measured to assess the effect of UT mix and vitamin D on the inflammatory response of ciPTEC-OAT1. Lipopolysaccharide (LPS) (10 μg/mL), which was used as a positive control, induced a 3-fold increase in IL-6 levels after 24 h exposure. Vitamin D, however, was able to reverse this pro-inflammatory effect of LPS by reducing the IL-6 levels. A 1.6-fold reduction was found for 100 nM and 500 nM, and a 1.8-fold reduction for 1 μM of $1,25(OH)_2D_3$ (Figure 3.5a). A 2-fold and 2.8-fold increase in IL-6 levels was observed following the exposure to 1× and 2.5× UT mix, respectively. Similar to what was observed for LPS, a small but evident trend in IL-6 level reduction was detected after co-treatment with $1,25(OH)_2D_3$ (Figure 3.5b,c). In all conditions, TNF-α levels measured were below the limit of detection.

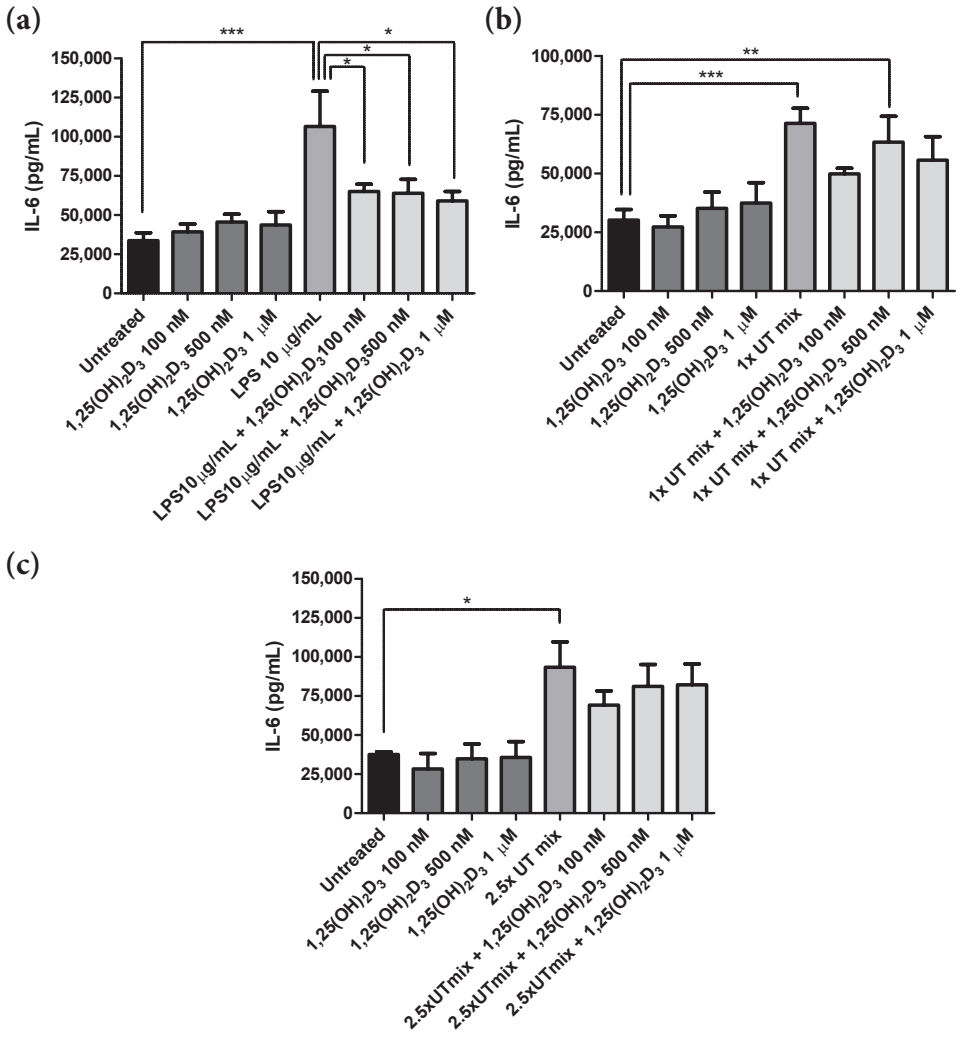


Figure 3.5. Vitamin D effect on IL-6 release by ciPTEC-OAT1 in inflammatory and uremic conditions. IL-6 release in cell culture supernatants after 24 h exposure to (**a**) $1,25(OH)_2D_3$ (100 nM, 500 nM and 1 μM), LPS (10 μg/mL) and combination of the two, (**b**) $1,25(OH)_2D_3$ (100 nM, 500 nM and 1 μM), 1× UT mix and combination of the two, (**c**) $1,25(OH)_2D_3$ (100 nM, 500 nM and 1 μM), 2.5× UT mix and their combination. Concentration expressed as pg/mL (mean ± SEM). At least three independent experiments were performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA followed by Dunnett's multiple comparison test, using as a control either untreated sample, LPS 10 μg/mL, 1× UT mix or 2.5× UT mix, as indicated).

Beneficial Effect of $1,25(OH)_2D_3$ on ciPTEC-OAT1 Epithelial Barrier Formation on HFM

In order to assess the stability of ciPTEC-OAT1 monolayer in uremic conditions and the effect of vitamin D on its tightness, cells were cultured on L-DOPA and collagen IV coated HFM, as described previously [30, 32]. Tight monolayer was confirmed by the presence of zonula occludens 1 (ZO-1) tight junction protein and actin staining (Figure 3.6a). Figure 3.6b shows inulin-FITC diffusion through HFM containing matured monolayers of ciPTEC-OAT1, untreated, exposed to 1 μM of $1,25(OH)_2D_3$, 2.5× UT mix or the combination of the two treatments for 24 h. UT mix increased inulin-FITC leakage to 813 ± 136 nmol·min^{-1}·cm^{-2} compared to the untreated fibers (400 ± 78 nmol·min^{-1}·cm^{-2}; $p < 0.01$). Simultaneous exposure to $1,25(OH)_2D_3$ could partially prevent the increase in inulin-FITC leakage induced by UT mix 2.5× (to 655 ± 85 nmol·min^{-1}·cm^{-2}). Figure 3.6c depicts a schematic presentation of inulin-FITC leakage in HFM carrying ciPTEC-OAT1 in examined conditions.

DISCUSSION

In the present study, we demonstrated the ability of ciPTEC-OAT1 to produce the most active form of vitamin D, $1,25(OH)_2D_3$, and its beneficial effect on various aspects of uremic conditions in ciPTEC-OAT1, including the protective effect on epithelial monolayer tightness. Considering the importance of the vitamin D deficiency often observed in CKD and ESRD, and the fact that vitamin D production is one of the main endocrine functions of proximal tubule cells, we were interested in determining whether ciPTEC-OAT1, intended for BAK purposes, possess all the necessary enzymes responsible for vitamin D metabolism. It has been shown that proximal tubule cells express 1α-hydroxylase, which is responsible for $25(OH)D_3$ conversion into $1,25(OH)_2D_3$, as well as CYP24A1, involved in $1,25(OH)_2D_3$ degradation [34]. Besides the proximal tubule, there are other, extra-renal sites expressing these enzymes and producing vitamin D ,such as the cells of the immune system (macrophages, monocytes, dendritic cells), and epithelial cells of the gastrointestinal tract, skin, breast, and lungs [35-41]. However, the major part of circulating vitamin D levels is kidney-derived, hence the severe deficiency is due to kidney failure [8]. We first evaluated the baseline expression of the genes for the activating and degrading enzymes and found that both are present in ciPTEC-OAT1, with a higher basal expression of the activating enzyme compared to the inactivating one. In line with the literature, following exposure to the active form of vitamin D, we observed a significant downregulation of 1α-hydroxylase and substantial upregulation of CYP24A1, confirming the existence of the negative feedback of $1,25(OH)_2D_3$ on its circulating concentration [42, 43]. Moreover, VDR is also expressed but not influenced by vitamin D treatment. Neither normal (1×) nor high (2.5×) concentrations of uremic toxins affected the expression of the enzymes and VDR, indicating that gene expression is not likely be altered in uremic conditions. In addition, we determined the actual conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$ in basic and uremic conditions. Cells were treated with a physiologic concentration of inactive vitamin D (100 nM, corresponding to 40 ng/mL in healthy individuals) and after 24 h

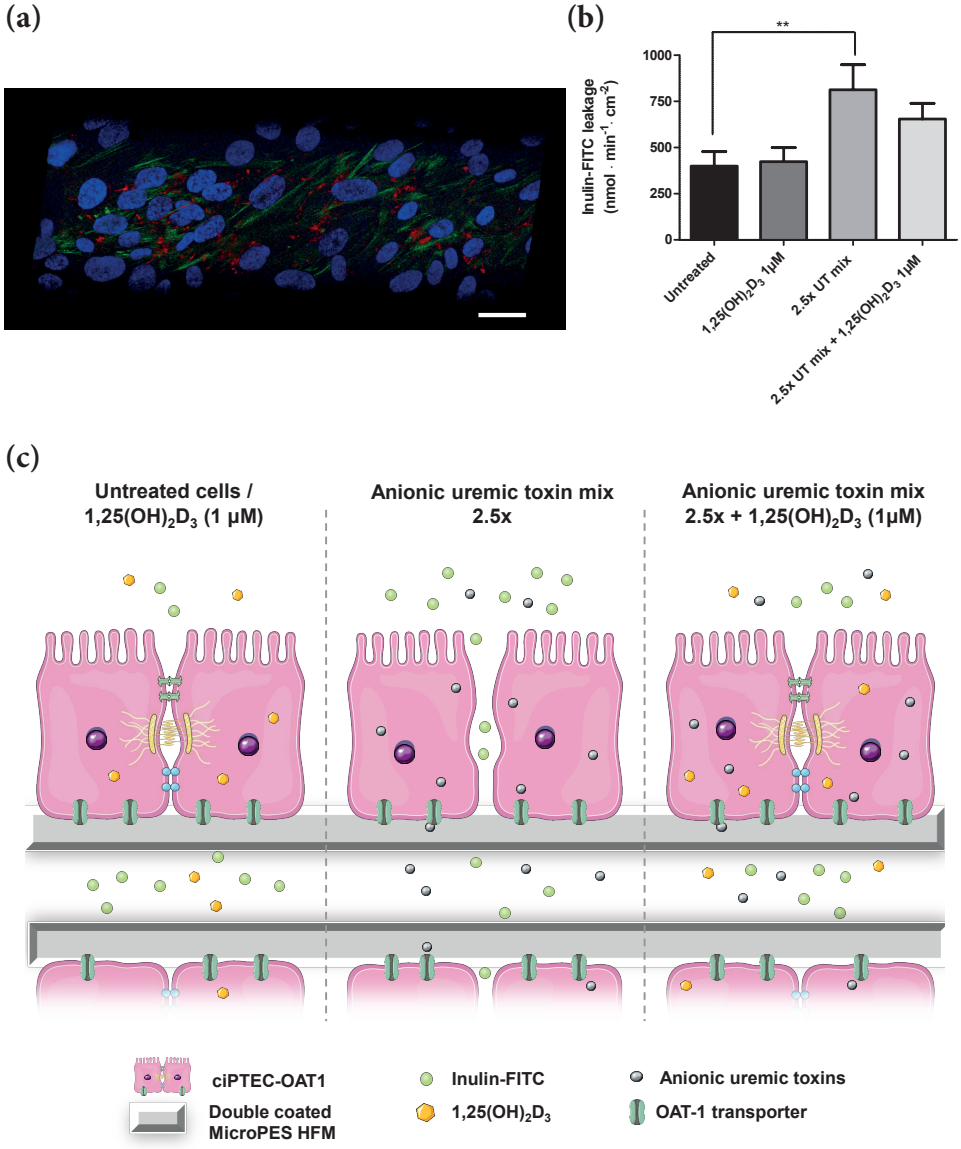


Figure 3.6. Transepithelial barrier function of ciPTEC-OAT1 cultured on hollow fiber membranes (HFM) in uremic conditions. (**a**) ciPTEC-OAT1 monolayer formation on L-DOPA (2 mg/mL) and collagen IV (25 µg/mL) coated HFM; ZO-1 expression (**red**), actin filaments (**green**) and nuclear staining with DAPI (**blue**) (scale bar 10 µm; 25× magnification); (**b**) transepithelial inulin-FITC diffusion in fibers containing untreated cells, cells exposed to $1,25(OH)_2D_3$ (1 µM), 2.5× UT mix and their combination for 24 h. Inulin-FITC diffusion is expressed as $nmol \cdot min^{-1} \cdot cm^{-2}$ (mean ± SEM). Two independent experiments were performed in triplicate. ** $p < 0.01$ (One-way ANOVA, Dunnett's multiple comparison test); (**c**) schematic presentation of inulin-FITC diffusion across HFM containing ciPTEC-OAT1 after various treatments.

the amount of active form of vitamin D generated was 32.5 pmol/L, corresponding to 13.5 pg/mL, which is slightly below the range of the active vitamin D serum levels in healthy patients [44, 45]. Although speculative, this indicates that ciPTEC-OAT1 may be able to sufficiently produce the active form of vitamin D. In accordance with gene expression levels in uremic conditions, we found that the conversion of $25(OH)D_3$ was not affected by uremic toxins, suggesting a normal endocrine function of ciPTEC-OAT1 in conditions relevant to BAK applications and kidney disease.

In addition to its well-described roles, such as maintenance of calcium homeostasis and mineralization, vitamin D is able to exert other, non-calciotropic effects [46]. Among the most relevant ones are certainly immunomodulatory actions [47, 48], with promotion of innate immune responses and the ability of immune system to fight infections [49-51], but also the suppression of the adaptive immune system with generation of tolerance, as shown for various auto-immune disorders (multiple sclerosis, type 1 diabetes, systemic lupus erythematosus and rheumatoid arthritis) [52-55]. Moreover, vitamin D is also involved in modulation of cell growth and proliferation, both in benign hyperplastic conditions and various cancer types [56].

In this study, we were particularly interested in the autocrine actions of vitamin D and therefore evaluated its effects on several cellular aspects of renal PTEC in uremic conditions. Initially, we observed that uremic toxins affect cell viability in a dose-dependent manner, while vitamin D did not have any effect. However, in the presence of anionic uremic toxins, especially at higher doses, vitamin D could restore cell viability. Numerous studies have described that some uremic toxins, such as indoxyl sulfate (IS), p-cresyl sulfate (pCS), and indole-3-acetic acid (IAA), are associated with increased inflammatory responses and oxidative stress both *in vitro* and *in vivo* [57-66]. To further address this, we measured IL-6 release by cells as an indication of inflammatory response, and ROS production, as a marker of oxidative stress, in uremic conditions and in the presence or absence of $1,25(OH)_2D_3$ in ciPTEC-OAT1. We found that uremic toxins do increase IL-6 secreted levels, as well as ROS intracellular generation. Moreover, our results indicate that $1,25(OH)_2D_3$ is able to reduce this increase in IL-6 levels and ROS production, confirming that vitamin D indeed has protective effects in uremic conditions, as suggested previously by *in vivo* studies, evaluating therapeutic effects of paricalcitol, a VDR activator, in uremic rats and hemodialysis patients [67-69].

A growing body of evidence suggests that vitamin D is essential for the correct functioning and maintenance of epithelial barriers, including gut mucosal barrier, corneal, pulmonary and kidney epithelial barriers, and its deficiency has been reported to promote barrier dysfunction and increased permeability [18, 70-72]. The key tight junction proteins responsible for a tight monolayer formation in kidney proximal tubule are claudin 2 and ZO-1 [73, 74]. We determined the effect of vitamin D on the stability of the proximal tubule epithelial monolayer in uremic conditions. For that purpose, ciPTEC-OAT1 were cultured on double-coated HFM to create kidney tubules consisting of mature epithelial cell monolayers, expressing both ZO-1 and claudin 2 (Figure 3.S4a). Interestingly, we observed increased barrier permeability in the presence of uremic toxins, as shown by inulin-FITC leakage. However, in the presence of vitamin D, a clear trend towards a smaller increase in inulin-FITC diffusion was detected, suggesting the protective effect

of vitamin D on the proximal tubule epithelial barrier integrity. Because the gene expression levels of ZO-1 and claudin 2 were not significantly influenced by uremic toxins or by vitamin D (Figure 3.S4b,c), we expect that the effect of vitamin D in attenuating epithelial barrier permeability might be due to a redistribution of tight junction proteins rather than an increased protein expression, as observed previously for the intestinal barrier [18].

The findings of the present study clearly support further development of BAK as a treatment modality in patients with ESRD. Extensive previous studies described an efficient way of culturing ciPTEC-OAT1 on double-coated HFM with the formation of tight epithelial monolayers, as well as the active transport activity of both OAT1 and OCT2, proteins responsible for the clearance of uremic waste metabolites [30, 32]. In addition, the lack of ciPTEC induced alloimmune response *in vitro* [75] and the successful upscaling of the BAK device [76] further encourage the use of these cells. Our current demonstration of the ability of the cells to activate and secrete the most active form of vitamin D is an additional important asset of the system. However, future studies should further investigate the choice of membranes used to support cell attachment, growth and monolayer formation, as this could potentially abolish the vitamin D activation function of ciPTEC-OAT1. It has been shown that some membrane types, in particular highly adsorptive and high cut-off membranes, could lead to a significant reduction in VDBP and $25(OH)D_3$ levels [6], potentially compromising the availability of $25(OH)D_3$ for megalin uptake and conversion by 1α-hydroxylase. For that reason, the polyethersulfone membranes used to support ciPTEC-OAT1 in the current settings should be tested for its suitability for use in BAK devices.

In conclusion, the ability of ciPTEC-OAT1 to produce active vitamin D could considerably boost BAK function, thus allowing the improvement of health status of kidney patients, not only by removing the excessive amounts of protein bound uremic toxins, but also by replicating one of the key endocrine functions of the proximal tubule. Eventually, the presence of $1,25(OH)_2D_3$ would greatly contribute to the maintenance of a strong epithelial monolayers for correct and efficient BAK function, and to improved mineral homeostasis and skeletal and cardiovascular health in CKD and ESRD patients. Future experiments will be designed to evaluate the safety and efficacy of a prototype BAK device *in vivo*, including the assessment of the beneficial effects of vitamin D as presented in this study.

ACKNOWLEDGMENTS

This research was supported by the Marie Curie ITN project BIOART (grant no. 316690, EU-FP7-PEOPLE-ITN-2012).

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SUPPLEMENTARY INFORMATION

(a)

Gene	Accession number	5'→3' forward primer	5'→3' reverse primer	Amplicon length
VDR	NM_000376.2	CTGACCCTGGAGACTTTGAC	TTCCTCTGCACTTCCTCATC	277
1α-hydroxylase	NM_000785.3	GGCAGAGTCTGAATTGCAAAT	CCGGGTCTTGGGTCTAACTG	97
CYP24A1	NM_000782.4	GGCCTCTTTCATCACAGAGCT	GCCTATCGCGACTACCGCAA	190

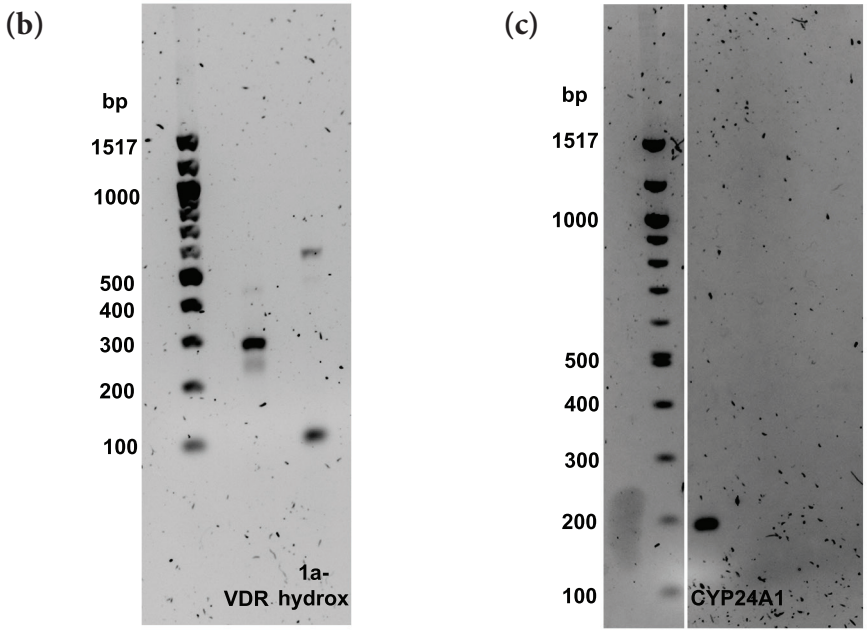


Figure 3.S1. VDR, 1 α-hydroxylase and CYP24A1 primers specificity. (**a**) Accession number, forward and reverse primer sequences and expected amplicon length of vitamin D receptor (VDR), 1α-hydroxylase and CYP24A1. (**b-c**) 1.5% agarose gel electrophoresis of PCR products of the three genes; all three PCR bands correspond to the expected amplicon length.

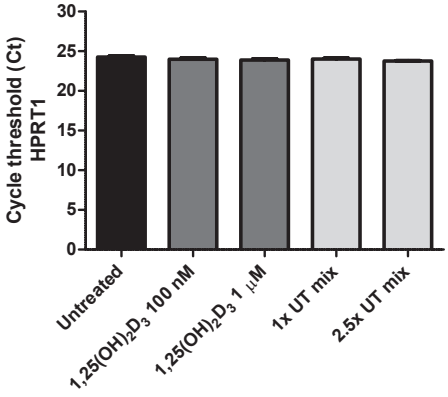


Figure 3.S2. Cycle threshold (Ct) values for HPRT1 in different experimental conditions used in the present study. HPRT1 expression was stable, with no significant differences between various treatments. Results obtained from three independent experiments performed in duplicate. One-way ANOVA followed by Tukey multiple comparison test was used for statistical analysis.

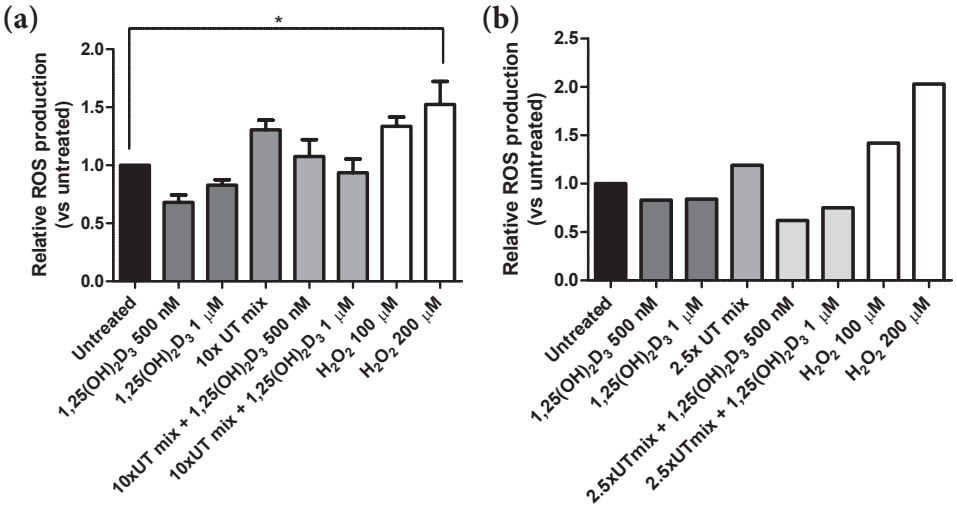
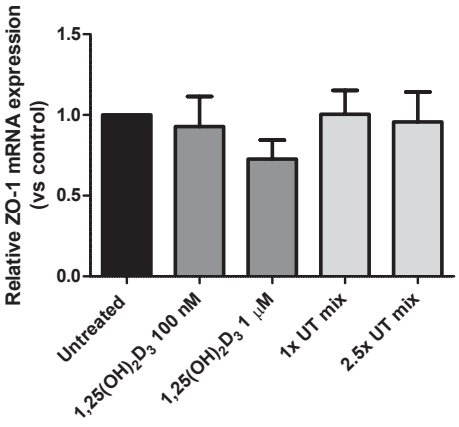


Figure 3.S3. Intracellular reactive oxygen species (ROS) production in ciPTEC-OAT1. (**a**) Relative ROS production in ciPTEC-OAT1 after 2 h exposure to $1,25(OH)_2D_3$ (500 nM and 1 μM), 10x UT mix, combination of the two at previous concentrations and H_2O_2 (100 μM and 200 μM). Three independent experiments were performed in duplicate. *p<0.05 (One-way ANOVA, Dunnett's multiple comparison test). (**b**) Relative ROS production in ciPTEC-OAT1 after 2 h exposure to $1,25(OH)_2D_3$ (500 nM and 1 μM), 2.5x UT mix, combination of the two at previous concentrations and H_2O_2 (100 μM and 200 μM).

(a)

	Ct (Mean ± SEM)		
ZO-1	26.3	±	0.16
Claudin 2	31.6	±	0.10

(b)



(c)

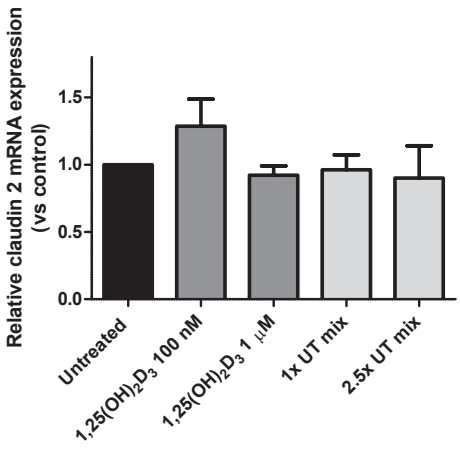


Figure 3.S4. Zonula occludens 1 (ZO-1) and claudin 2 expression in ciPTEC-OAT1. (**a**) Cycle threshold (Ct) values (expressed as mean ± SEM) reflecting ZO-1 and claudin 2 expression levels in basal conditions. Relative mRNA expression of (**b**) ZO-1 and (**c**) claudin 2 in ciPTEC-OAT1 following 24 h exposure to 100 nM and 1 μM of $1,25(OH)_2D_3$ or 1x and 2.5x UT mix, compared to control (untreated ciPTEC-OAT1). Three independent experiments were performed in duplicate. One-way ANOVA followed by Dunnett's multiple comparison test was used for statistical analysis, and no differences were found between examined conditions.

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Scientific Reports. 2017; 7(1):7103
doi:10.1038/s41598-017-07582-1

CHAPTER IV

ALLOSTIMULATORY CAPACITY OF CONDITIONALLY IMMORTALIZED PROXIMAL TUBULE CELL LINES FOR BIOARTIFICIAL KIDNEY APPLICATION

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ABSTRACT

Novel renal replacement therapies, such as a bioartificial kidney (BAK), are needed to improve current hemodialysis treatment of end-stage renal disease (ESRD) patients. As BAK applications may reveal safety concerns, we assessed the alloimmunization and related safety aspects of readily available conditionally immortalized human proximal tubule epithelial cell (ciPTEC) lines to be used in BAK. Two ciPTEC lines, originally derived from urine and kidney tissue, were characterized for the expression and secretion of relevant molecules involved in alloimmunization and inflammatory responses, such as HLA class-I, HLA-DR, CD40, CD80, CD86, as wells as soluble HLA class I and proinflammatory cytokines (IL-6, IL-8 and TNF-α). A lack of direct immunogenic effect of ciPTEC was shown in co-culture experiments with peripheral blood mononuclear cells (PBMC), after appropriate stimulation of ciPTEC. Tight epithelial cell monolayer formation on polyethersulfone flat membranes was confirmed by zonula occludens-1 (ZO-1) expression in the ciPTEC tight junctions, and by restricted inulin-FITC diffusion. Co-culture with (activated) PBMC did not jeopardize the transepithelial barrier function of ciPTEC. In conclusion, the absence of allostimulatory effects and the stability of ciPTEC monolayers show that these unique cells could represent a safe option for BAK engineering application.

Keywords: bioartificial kidney; conditionally immortalized proximal tubule cells; chronic kidney disease; end-stage renal disease; alloimmune response

INTRODUCTION

End-stage renal disease (ESRD) is the final and most severe stage of chronic kidney disease (CKD). It has been estimated that almost 10% of the population worldwide is affected by CKD. The major problem in CKD patients, beside the loss of kidney function, is the concomitant presence of various comorbidities, especially cardiovascular disorders. These develop over time as a result of longstanding hypertension, disturbances in calcium-phosphate metabolism, and constant accumulation of uremic metabolites, and result in increased mortality within the CKD population [1-3]. Currently available treatment options for patients with ESRD are hemodialysis, peritoneal dialysis and kidney transplantation, the latter one being preferred since the ability to restore kidney function is associated with a better life expectancy and a higher quality of life. Unfortunately, for many ESRD patients this treatment is not readily available because of organ shortage, which keeps these patients dependent on dialysis. However, dialysis is not very efficient in removing the uremic waste products, especially the protein-bound and larger size molecules, maintaining the progression of most of the mortality-associated comorbidities [4].

Therefore, novel therapies for CKD are needed, and one of the most promising options is the bioartificial kidney (BAK) device, composed of proximal tubule epithelial cells (PTEC) cultured on hollow fiber membranes (HFM) with formation of confluent, fully differentiated epithelial monolayers [5]. The reason why PTEC are especially attractive for such an application is that these cells are specialized in the excretion of many xenobiotics, including the endogenous uremic

waste compounds (also named uremic toxins). In particular, PTEC can help to excrete protein-bound toxins, which cannot be removed by standard dialysis treatments. Recent work from our group showed that PTEC cultured on HFM are able to take up and excrete indoxyl sulfate and kynurenic acid, two prototypical protein-bound uremic toxins [6].

One of the crucial issues to take into consideration when developing a BAK is sufficient availability of suitable cells. We developed conditionally immortalized proximal tubule epithelial cell lines (ciPTEC), derived from human urine or kidney tissue, as an unlimited and invariable cell source for BAK application [7,8]. CiPTEC were immortalized with the temperature-sensitive mutant U19tsA58 of SV40 large T antigen (SV40T) and the essential catalytic subunit of human telomerase (hTERT), as described [9-11]. This allows the cells to proliferate at the permissive temperature of 33°C and to fully differentiate to mature PTEC at non-permissive temperature of 37°C. CiPTEC were extensively characterized for most proximal tubule functions such as reabsorption and excretory transport activities and successfully cultured on biofunctionalized HFM [6-8,12].

Many of the previous studies concerning BAK have focused on the immunomodulatory function of renal tubular cells, in particular reduction of proinflammatory and increase of anti-inflammatory cytokines plasma and serum levels [13-15]. In the present study, though, we evaluated the *in vitro* immunosafety of ciPTEC for BAK application, with particular attention to their direct allogeneic effect. To that purpose, we thoroughly characterized the expression and release of Human Leukocyte Antigens (HLA), as well as the expression of several co-stimulatory ligands on two ciPTEC lines, one originally derived from healthy donor urine and one from kidney tissue [7,8]. In addition, we assessed the ability of ciPTEC to mediate an inflammatory response by measuring the production of relevant proinflammatory mediators, like Interleukin 6 (IL-6), Tumor Necrosis Factor α (TNF-α), and Interleukin 8 (IL-8), in various stimulatory conditions. In order to determine the direct immunogenic effect of ciPTEC, co-culture experiments with immune cells were performed. Finally, the paracrine effect of immune cells on ciPTEC monolayer integrity was examined as well. For this, cells were grown on flat biofunctionalized polyethersulfone membranes, with a Mw cut-off of 50 kDa, as prototypical component of a BAK device [6,16-19].

MATERIALS AND METHODS

Ethics statement

The ethics committee of the University Medical Center Utrecht on research involving human subjects approved this study, and written informed consent was obtained from each patient and each healthy volunteer. All experiments were performed in accordance with relevant guidelines and regulations.

Cell culture

Two previously established ciPTEC lines, derived from a healthy volunteer urine sample (ciPTEC-U) and tissue from a non-transplanted donor kidney (ciPTEC-T1) [7,8], were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1 DMEM/F-12) (Gibco, Life Technologies, Paisly, UK) supplemented with 10% (v/v) fetal calf serum (FCS) (Greiner Bio-One, Alphen aan den Rijn, the Netherlands), 1% (v/v) penicillin/streptomycin (Sigma Aldrich, Zwijndrecht, the Netherlands), 5 μg/mL insulin, 5 μg/mL transferrin, 5 μg/mL selenium, 35 ng/mL hydrocortisone, 10 ng/mL epidermal growth factor and 40 pg/mL tri-iodothyronine (Sigma Aldrich, Zwijndrecht, the Netherlands). CiPTEC-U were cultured up to 50 and ciPTEC-T1 up to 45 passages. Cells were cultured at 33°C and 5% (v/v) CO_2 to allow proliferation, given that they were immortalized with SV40T. Prior to the experiments, cells were seeded at appropriate density, which was 48,000 cell/cm^2 and 19,250 cell/cm^2 for ciPTEC-U and ciPTEC-T1, respectively. Cells were grown for 1 day at 33°C, 5% (v/v) CO_2 to allow them to adhere, then cultured at 37°C, 5% (v/v) CO_2 for differentiation and maturation, changing the medium every second day. After 7 days of maturation, cells were treated with chemicals where indicated and used in experiments. The following treatments were used depending on the experimental set-up: interferon-γ (IFN-γ) 150-600 ng/ml, lipopolysaccharide (LPS) (*Escherichia coli* 0127:B8) 10 μg/ml, indoxyl sulfate 1-2 mM (all from Sigma Aldrich, Zwijndrecht, the Netherlands) for 48 h.

HLA typing

HLA allele typing of ciPTEC cell lines was performed by the Luminex method using the LABtype SSO kit (OneLambda, Canoga Park, USA) according to the manufacturer's instructions.

Enzyme-Linked Immuno Sorbent Assays

The production of IL-6, TNF-α, IL-8 and soluble HLA class I (sHLA class I) molecules in various stimulatory conditions, was measured by means of Enzyme-Linked Immuno Sorbent Assays (ELISAs). Cell culture supernatants were collected after exposure to various stimulatory conditions, centrifuged for 10 min at 240 x g, 4°C, and stored at -80°C. DuoSet® ELISA Development Systems kits (IL-6 #DY206, TNF-α #DY210, IL-8 #DY208; R&D systems, Abingdon, UK) and HLA class I kit (Proteintech, Chicago, IL, USA) were used to quantify the cytokine and sHLA class I levels respectively, in complete cell culture medium supernatants according to manufacturer's instructions. The optical density was determined immediately using the iMark Microplate Absorbance Reader (Bio-Rad, Japan) set to 450 nm. Each sample was measured in duplicates and quantification was done using Microplate Manager software (version 6.0, Bio-Rad Laboratories, Hercules, CA, USA) capable of generating a four parameter logistic (4-PL) curve-fit.

Flow cytometry

The surface expression of HLA class I, HLA-DR, CD40, CD80 and CD86 molecules was evaluated using flow cytometry. First, ciPTEC were cultured until confluency (7 day maturation at 37°C, 5% (v/v) CO_2), followed by the stimulation with IFN-γ 150-600 ng/ml, LPS 10 μg/

ml, indoxyl sulfate 1-2 mM, or conditioned medium derived from either resting or CD3/CD28-activated human peripheral blood mononuclear cells (PBMC). Next, cells were washed once with Hank's Balanced Salt Solution (HBSS; Gibco, Life Technologies, Paisly, UK), detached with Accutase® solution (Sigma Aldrich, Zwijndrecht, the Netherlands), centrifuged at 240 x g for 5 min, washed twice with HBSS and incubated with 0.05% (v/v) viability dye (Fixable Viability Dye eFluor 780; eBioscience, San Diego, CA, USA) in protein-free Phosphate Buffered Saline (PBS) (Lonza, Verviers, Belgium) for 30 min at 4°C, protected from light. After two washing steps with FACS buffer, an 1% (w/v) solution of Bovine Serum Albumin (BSA) (Roche Diagnostics, Mannheim, Germany) in PBS, cell pellets were fixated with ice-cold 4% (w/v) paraformaldehyde in PBS for 10 min on ice, washed, centrifuged at 240 x g for 5 min and incubated with the following antibodies diluted in FACS buffer at appropriate concentration for 30 min at room temperature (RT): anti-human HLA-DR-PE (clone LN3) (1:50), anti-human CD40-FITC (clone 5C3) (1:100), anti-human CD80(B7-1)-PE (clone 2D10.4) (1:50), anti-human CD86(B7-2)-PE (clone IT2.2) (1:20) (antibodies were purchased from eBiosciences, San Diego, CA, USA) and W6/32 for detection of human HLA class I molecules (1:200) [20]. In case of W6/32 antibody, after a washing step with FACS buffer, cell pellets were incubated with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG 1:200; Molecular probes, Eugene, OR, USA) for another 30 min in dark. Finally, after the staining procedure, cell pellets were washed and resuspended in 100-150 µl of FACS buffer and the expression of surface markers was measured using flow cytometry (BD FACSCanto II, BD Biosciences, San Jose, CA, USA). A minimum of 10,000 cells per sample were counted and data were analyzed using FlowLogic software (600.0A; Inivai Technologies, Mentone, Victoria, Australia). Dead cells were excluded from analysis and median fluorescent intensity (MFI) was used to quantify the expression where possible.

Isolation of peripheral blood mononuclear cells

Human PBMC were isolated from buffy coats of healthy donors (Sanquin, Amsterdam, the Netherlands) using Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany). Briefly, blood was diluted 1:1 in PBS containing 2% (v/v) FCS, poured in Leucosep tubes and centrifuged (1,000 x g, 13 min). The enriched cell fraction containing PBMC was harvested and washed three times with PBS/2% (v/v) FCS. Remaining erythrocytes were lysed by adding 5 mL of cold lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in 500 mL demi water, pH adjusted to 7.4 and filter sterilized) for 5 min on ice. Afterwards, PBMC were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 1 mM sodium pyruvate (Sigma Aldrich, Zwijndrecht, the Netherlands). Conditioned medium from PBMC was collected after 24 h of either unstimulated cells, or cells stimulated with anti-CD3 (clone CLB-T3/2) and anti-CD28 (clone CLB-CD28) antibodies (both 1:10,000, Sanquin), and used diluted 1:1 with RPMI 1640 medium.

Direct co-culture of ciPTEC and PBMC

Following 7 days of maturation at 37°C, 5% (v/v) CO_2, ciPTEC were stimulated with IFN-γ, LPS or exposed to conditioned medium from CD3/CD28-activated PBMC for 48 h. Prior to the co-culture, freshly isolated PBMC were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE or CFSE) (Vybrant® CFDA-SE Cell Tracer Kit, Life Technologies, Eugene, OR, USA), 5 μM final concentration. In brief, the staining was done in PBS for 10 min, at RT, in dark. The reaction was quenched with ice-cold FCS, after which the cells were washed three times with PBS/2% (v/v) FCS and resuspended in RPMI 1640. In the meantime, ciPTEC were rinsed with HBSS, detached using Accutase® solution and resuspended at appropriate concentration in RPMI 1640. CiPTEC-PBMC co-cultures were performed in 96 well U-bottom cell culture plates (Greiner Bio-One, Frickenhausen, Germany) at different ratios (1:5, 1:15 and 1:30 - ciPTEC:PBMC) for at least 5 days. As positive controls CFSE-labelled PBMC were stimulated with concanavalin A (ConA) (Sigma Aldrich, Zwijndrecht, the Netherlands) 5 μg/ml, phytohemagglutinin-P (PHA-P) (Sigma Aldrich, Zwijndrecht, the Netherlands) 5 μg/ml, or with human T-Activator CD3/CD28 dynabeads (Gibco, Thermo Fisher Scientific, Vilnius, Lithuania), at a ratio of one bead per cell. Proliferation of CFSE-labelled PBMC was assessed using flow cytometry by measuring dilution of CFSE and therefore cell division [21]. Data were analyzed using FlowLogic software and presented as percentage of cells in the divided population [22].

Cell culture on microPES and co-culture of ciPTEC-T1 and PBMC

MicroPES (polyethersulfone) type 2F iv hydrophilic flat membranes (thickness 110±10 μm) were purchased from 3M Deutschland GmbH (Wuppertal, Germany). For cell culture, round-shaped pieces of the microPES membranes (diameter 12 mm, surface growth area 1.12 cm^2) were cut from the flat sheets, mounted on empty Transwell®(Corning Costar, NY, USA) membrane support systems using custom-made sealing rings [23], sterilized with 0.2% (v/v) solution of peracetic acid (Sigma Aldrich, Zwijndrecht, the Netherlands) in 4% (v/v) ethanol for 30 min, and then extensively rinsed with HBSS. Afterwards, the double coating was applied on the membranes to support cell attachment and growth, based on previously published studies [12,17]. First, L-DOPA (L-3,4-dihydroxyphenylalanine, Sigma Aldrich, Zwijndrecht, the Netherlands) was dissolved in 10 mM Tris buffer (pH 8.5) at 37°C for 45 min with occasional mixing, at 2 mg/ml final concentration, as described previously [6,12,24], filter sterilized and applied on the membranes for 4 h at 37°C. The second coating was provided by 25 μg/ml solution of collagen IV (Sigma Aldrich, Zwijndrecht, the Netherlands), for 1 h at 37°C. Following the coating procedure, microPES membranes were washed in HBSS and used further for cell seeding.

CiPTEC-T1 were seeded on double-coated membranes, in the apical compartment of the Transwell® system, at 90,000 cells/cm^2. After initial proliferation at 33°C for 1 day, and 7 days maturation at 37°C, the indirect co-culture of 48 h was performed with freshly isolated PBMC which were seeded in the basolateral compartment (2 million cells/ml), and exposed to anti-CD3 and anti-CD28 antibodies (both 1:10,000), LPS (1 μg/ml), or human plasma pooled from healthy donors and from CKD patients (10% (v/v) or 20% (v/v), diluted with culture medium).

Cell monolayer integrity

Following 48 h indirect co-culture, PBMC were removed and ciPTEC-T1 cell monolayer barrier function on double-coated microPES membranes was assessed by quantifying diffusion of inulin-fluorescein isothiocyanate (FITC) (Sigma Aldrich, Zwijndrecht, the Netherlands) (0.1 mg/ml in Krebs-Henseleit (KH) buffer (Sigma Aldrich, Zwijndrecht, the Netherlands) supplemented with 10 mM HEPES (Acros Organics, New Jersey, USA)) from basolateral to apical compartment for 1 h at 37°C. Coated and non-coated membranes without cells were used as controls. Apical and basolateral exposure of ciPTEC-T1 to cisplatin (Sigma Aldrich, Zwijndrecht, the Netherlands; 50 μM) for 24 h was used as a positive control for inulin-FITC leakage. Fluorescence was measured at excitation wavelength of 492 nm and emission wavelength of 518 nm, by means of fluorescent platereader (Fluoroskan Ascent FL, Labsystems). Measured fluorescence values were used to calculate inulin-FITC concentration in apical compartment of all samples. Finally, inulin-FITC diffusion (J) was calculated as described previously [12], using the following formula:

$$J = ((((C/Mw) * V) * 10^{9})/t)/A = pmol \cdot min^{-1} \cdot cm^{-2}$$

where C is the apical concentration of inulin-FITC in mg/ml, Mw the average inulin-FITC molecular weight (4,500 mg/mmol), V the volume in the apical compartment (0.5 ml), t the time (60 min) and A the surface area of microPES membranes (1.12 cm^2).

Immunocytochemistry

The expression of zonula occludens-1 (ZO-1) on ciPTEC-T1 monolayers cultured on microPES membranes was assessed using a slightly modified immunocytochemistry procedure described by Jansen *et al.* [12]. In brief, cells were washed with HBSS two times, fixed with 2% (w/v) paraformaldehyde in PBS containing 4% (w/v) sucrose (Sigma Aldrich, Zwijndrecht, the Netherlands) for 5 min, then washed three times with 0.1% (v/v) Tween (Sigma Aldrich, Zwijndrecht, the Netherlands) solution in PBS and permeabilized with 0.3% (v/v) Triton (Merck, Darmstadt, Germany) solution. After another three washing steps with 0.1% (v/v) Tween-PBS, cells were exposed to Dako Target Retrieval solution (DAKO, Carpinteria, CA, USA) for 1 h, according to manufacturer's instructions. Next, cells were incubated with DAKO Protein Block serum-free solution (DAKO, Carpinteria, CA, USA) for 1 h, followed by the application of rabbit anti-human ZO-1 antibody (Invitrogen, Carlsbad, CA, USA) (1:400 in DAKO Antibody Diluent (DAKO, Carpinteria, CA, USA)) for 1 h at RT. After extensive washing with 0.1% (v/v) Tween-PBS, secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG 1:200 in DAKO Antibody Diluent; Life Technologies, Eugene, OR, USA) was applied for 1 h at RT. Finally, membranes were mounted on glass slides using ProLong Gold antifade reagent containing DAPI (Life Technologies, Eugene, OR, USA) and cells were imaged using confocal microscope (Leica TCS SP8 X, Leica Microsystems CMS GmbH, Wetzlar, Germany) and analyzed using Leica Application Suite X software (Leica Microsystems CMS GmbH).

Data analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test or, where appropriate, Tukey's multiple comparison test, and a p-value <0.05 was considered significant. Software used for statistical analysis was GraphPad Prism (version 5.03; GraphPad software, La Jolla, CA, USA). All experiments were repeated independently at least three times, unless stated differently.

RESULTS

HLA class I expression and release by ciPTEC-U and –T1 lines

The HLA type of the two ciPTEC lines was determined by PCR-SSO. The results are summarized in Table 4.S1. Next, the surface expression of HLA class I molecules was evaluated in both ciPTEC-U and –T1 lines in basal, as well as in several stimulatory conditions, using W6/32 monoclonal antibody directed against the core of HLA-A, B and C antigens. Both cell lines showed the expression of HLA class I molecules in untreated conditions. In ciPTEC-U, the HLA class I expression was increased after stimulation with IFN-γ at 300 ng/ml for 48 h (21 ± 4%, $p<0.05$). In ciPTEC-T1 the HLA class I expression was enhanced by IFN-γ at 300 and 600 ng/ml (63 ± 7% and 80 ± 22%, respectively, $p<0.001$) and by LPS 10 µg/ml (54 ± 7%, $p<0.001$). The expression of HLA class I molecules was not significantly upregulated after 48 h stimulation with indoxyl sulfate (1 and 2 mM), or conditioned medium (CM) from PBMC (resting and CD3/CD28 activated) (Figure 4.1).

In addition to surface expression, the release of soluble forms of HLA class I molecules in cell culture supernatants was also examined. We found low concentrations of sHLA class I in the supernatant of untreated cells, with a significant increase after stimulation with IFN-γ (300 ng/ml) in ciPTEC-U, and a similar trend in ciPTEC-T1 (Figure 4.2). No increase in sHLA class I concentration was observed following LPS or indoxyl sulfate treatment.

The expression of HLA class II and co-stimulatory molecules

HLA-DR expression was measured as an indicator of the presence of HLA class II molecules on ciPTEC. In both ciPTEC-U and –T1, the expression of HLA-DR was undetectable in control conditions. Treatment with conditioned medium from activated PBMC (activated PBMC-CM) was the only tested stimulatory condition able to induce the expression of HLA-DR. Following 48 h exposure to activated PBMC-CM, 22 ± 7% of ciPTEC-U and 21 ± 4% of ciPTEC-T1 stained positive with anti HLA-DR antibody (Figure 4.3). Other molecules that are important for the immunogenicity of allogeneic tissues and cells are the co-stimulatory ligands CD40, CD80 and CD86. We found a low expression of CD40 on both cell lines, as compared to monocytes which were used as positive controls. Again, the only condition tested able to induce an increase in CD40 expression was the activated PBMC-CM (Figure 4.3). The expression of CD80 and CD86 was either very low or undetectable in all conditions examined.

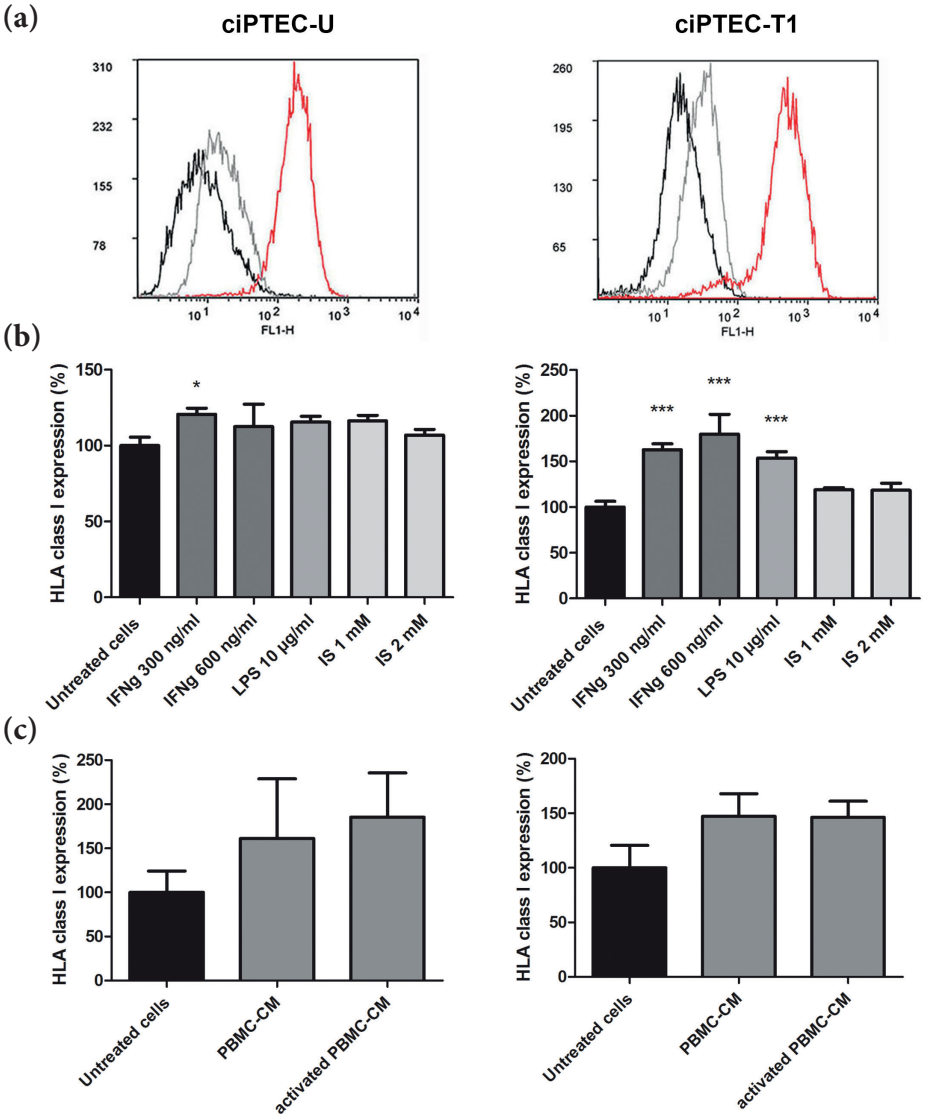


Figure 4.1. HLA class I expression in ciPTEC in various stimulatory conditions. (**a**) Representative histograms of HLA class I expression from flow cytometric analysis. Black histograms correspond to the unstained cells, grey to the negative control (absence of primary monoclonal W6/32 antibody), and red represent basal levels of HLA class I expression. (**b**) Effect of IFN-γ (300 and 600 ng/ml), LPS (10 µg/ml) and indoxyl sulfate (IS) (1 and 2 mM) on HLA class I expression in ciPTEC-U and -T1. Treatments were performed for 48 h prior to flow cytometric measurements. (**c**) Effect of conditioned medium derived from resting (PBMC-CM) and aCD3/aCD28 activated (activated PBMC-CM) PBMC on the expression of HLA class I after 48 h exposure. Expression is shown as %, based on median fluorescence intensity (MFI) and SEM values of three independent experiments performed in duplicate. *p<0.05, ***p<0.001, compared to untreated cells (One-way ANOVA, Dunnett's multiple comparison test).

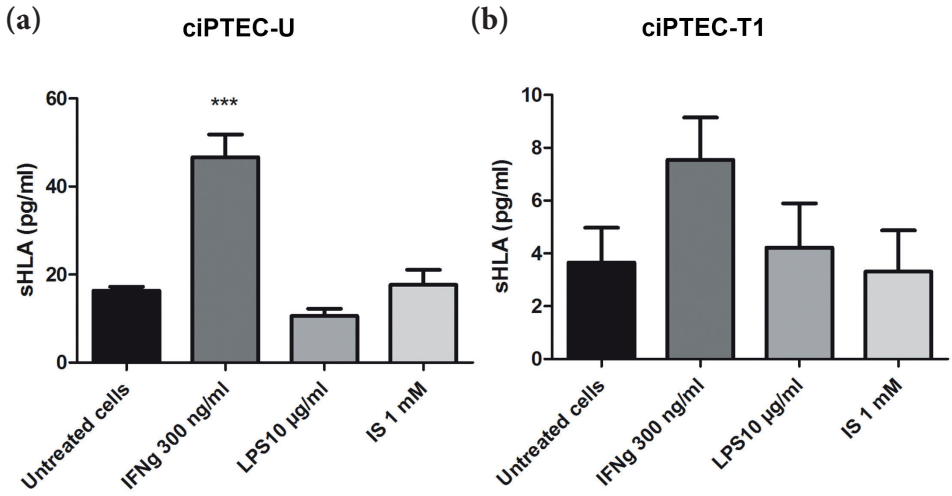


Figure 4.2. Release of soluble HLA (sHLA) class I antigens by ciPTEC. Secretion of sHLA class I molecules in cell culture supernatant following 48 h stimulation with IFN-γ (300 ng/ml), LPS (10 µg/ml) and indoxyl sulfate (IS) (1 mM) by (**a**) ciPTEC-U and (**b**) ciPTEC-T1, measured using ELISA assay and expressed as pg/ml (mean ± SEM). Two independent experiments were performed in duplicate. ***p<0.001 compared to untreated cells (One-way ANOVA, Dunnett's multiple comparison test).

IV

Lack of allostimulation by ciPTEC in direct co-culture with PBMC

The ability of ciPTEC to induce an alloimmune response was evaluated by measuring the proliferation of freshly isolated human PBMC (obtained from random blood donors) in direct co-culture experiments. The proliferative potential of the PBMC was convincingly shown after stimulation with ConA (5 µg/ml), PHA-P (5 µg/ml) and human T-Activator CD3/CD28 dynabeads (Figures 4.4a and 4.4c). Notably, both ciPTEC lines failed to induce any proliferation of PBMC after 5 days of culture, irrespective of the pre-stimulation treatment of ciPTEC and ratios of ciPTEC to PBMC (Figures 4.4b and 4.4c).

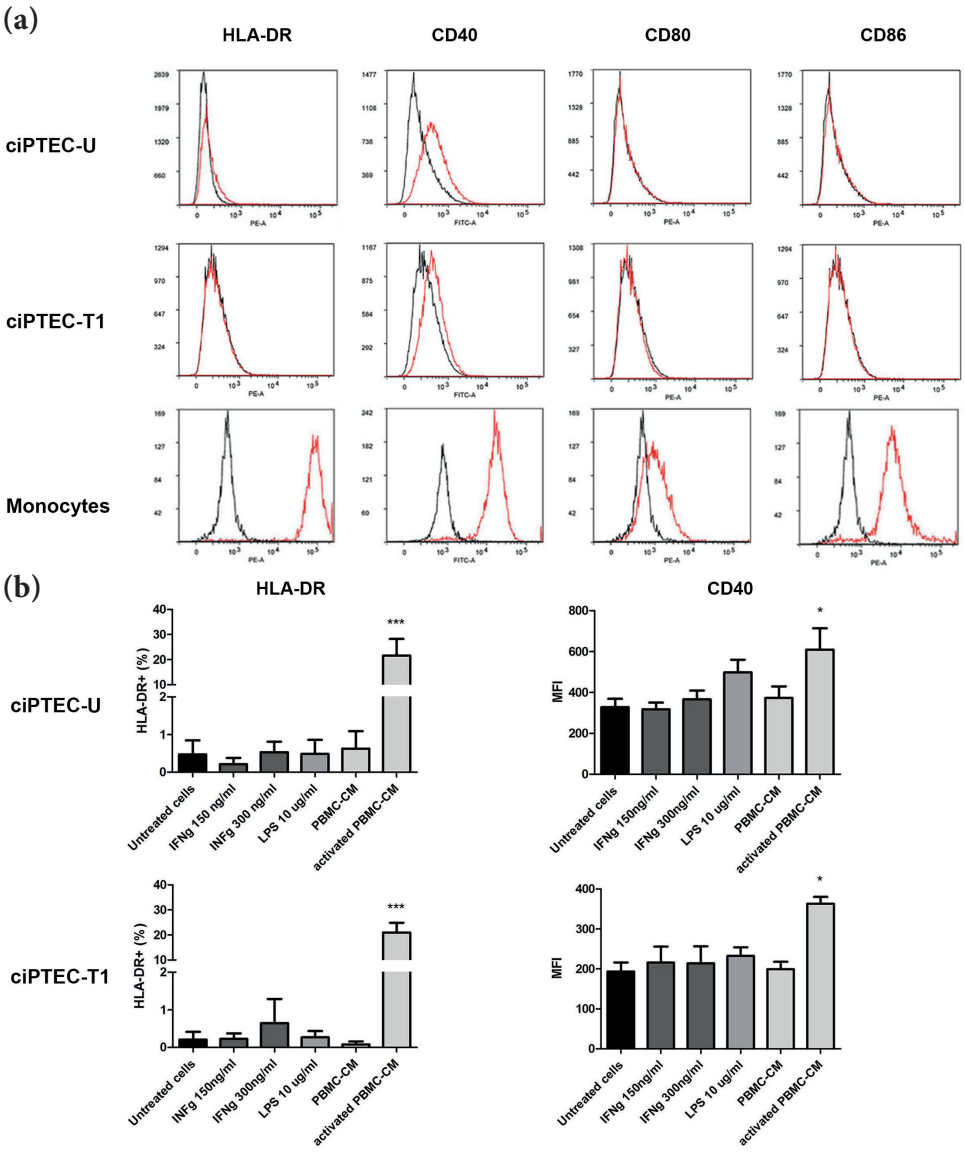
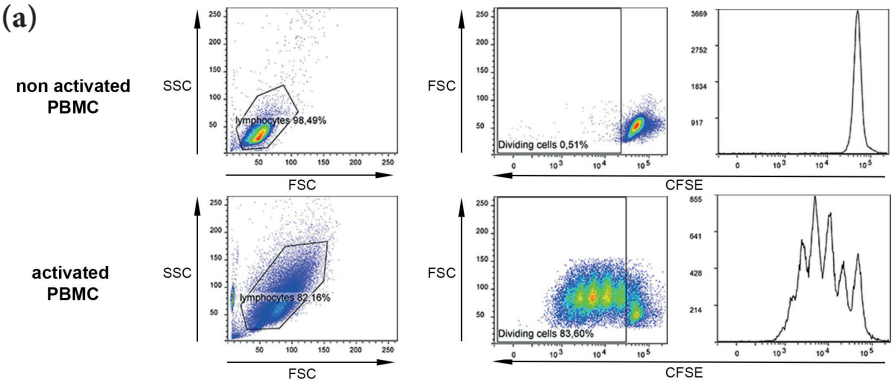
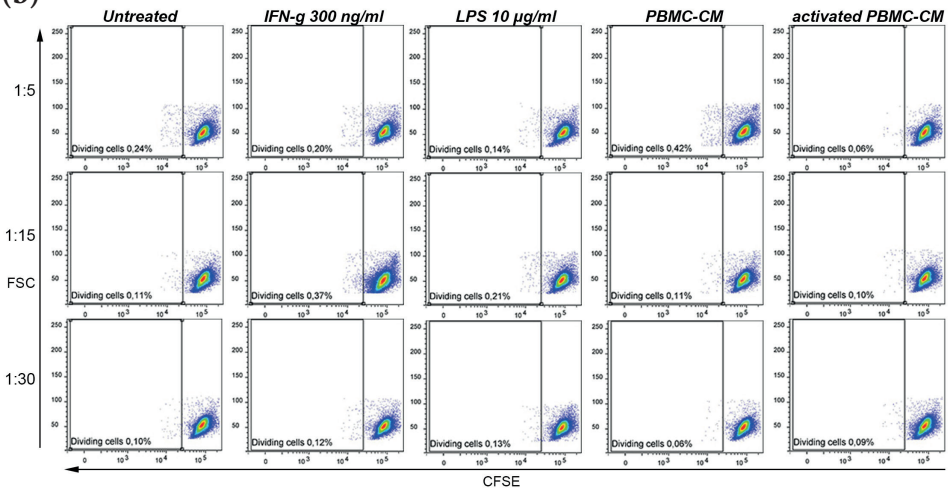


Figure 4.3. Expression of co-stimulatory molecules in ciPTEC. (**a**) Representative histograms from flow cytometric analysis of HLA-DR, CD40, CD80 and CD86 in ciPTEC-U, -T1 and monocytes (used here as a cell type positive for all four surface markers evaluated). In all histogram plots black color represents blank (unstained cells) and red color represents basal expression of either HLA-DR, CD40, CD80 or CD86. (**b**) Quantification of HLA-DR and CD40 expression in ciPTEC cell lines, expressed as % of positive cells ± SEM and MFI ± SEM respectively. Three independent experiments were performed. *p<0.05, ***p<0.001, compared to untreated control (One-way ANOVA, Dunnett's multiple comparison test).

(a)



(b)



(c)

Co-culture	ciPTEC-U:PBMC ratio			ciPTEC-T1:PBMC ratio		
	1:5	1:15	1:30	1:5	1:15	1:30
	% of dividing cells (mean ± SEM)					
Pre-stimulation of ciPTEC:						
Untreated	2.4 6 ± 0.99	2.22 ± 1.29	1.38 ± 0.64	2.11 ± 1.59	0.53 ± 0.24	0.62 ± 0.31
IFN-γ 150 ng/ml	2.11 ± 0.83	1.92 ± 0.98	1.48 ± 0.57	2.45 ± 1.97	0.49 ± 0.23	0.69 ± 0.41
IFN-γ 300 ng/ml	2.42 ± 1.14	2.47 ± 1.27	1.27 ± 0.48	2.23 ± 1.82	0.52 ± 0.25	0.86 ± 0.48
LPS 10 µg/ml	2.02 ± 0.48	1.80 ± 0.88	1.20 ± 0.54	1.77 ± 1.41	0.51 ± 0.21	1.09 ± 0.73
PBMC-CM	2.79 ± 1.20	2.58 ± 1.24	1.6 ± 0.66	0.52 ± 0.41	0.65 ± 0.34	0.93 ± 0.51
Activated PBMC-CM	2.16 ± 1.03	2.40 ± 1.34	1.5 ± 0.62	0.34 ± 0.29	0.53 ± 0.26	0.91 ± 0.42

Controls	PBMC
	% of dividing cells (mean ± SEM)
Stimulations:	
Untreated	1.88 ± 0.36
ConA 5 µg/ml	83.43 ± 0.86 *
PHA 5 µg/ml	71.43 ± 0.78 *
Dynabeads (aCD3/aCD28)	83.45 ± 1.84 *

Figure 4.4. Immunogenic effect of ciPTEC in direct co-culture with PBMC. (**a**) Representative dot plots and histograms of lymphocyte proliferation assays that were performed using carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled PBMC either untreated or activated with human T-Activator CD3/CD28 dynabeads (1 bead per cell). (**b**) Representative dot plots of CFSE-labeled PBMC proliferation after 5 days of co-culture with ciPTEC-U (untreated or pre-stimulated in various ways) in ratios 1:5, 1:15 or 1:30. Comparable dot plots were observed for the co-culture of PBMC and ciPTEC-T1. (**c**) PBMC proliferation after 5 days of co-culture in various ratios with untreated or pre-stimulated ciPTEC-U and –T1 cells, expressed as % of dividing cells. There were no differences in proliferation of PBMC from co-culture compared to untreated PBMC. Four independent co-culture experiments for ciPTEC-U and three for ciPTEC-T1 were performed. *p<0.001 (One-way ANOVA, Tukey's multiple comparison test) for PBMC proliferation induced by ConA (5 μg/ml), PHA-P (5 μg/ml) or T-Activator CD3/CD28 dynabeads (1 bead per cell) compared to untreated PBMC.

Release of proinflammatory mediators by ciPTEC-U and –T1

To assess whether ciPTEC are able to mediate an inflammatory response, the release of IL-6, IL-8 and TNF-α was determined in cell culture supernatants (Figure 4.5). In basal conditions, there was a low secretion of IL-6 and IL-8 by both cell lines, and of TNF-α by ciPTEC-U. Exposure to LPS 10 μg/ml significantly increased the secretion of all three cytokines. A moderate increase of IL-6 secretion was also observed after stimulation with indoxyl sulfate. Remarkably, TNF-α levels in supernatants of ciPTEC-U were significantly decreased after IFN-γ exposure.

Strong ciPTEC barrier formation on microPES membranes is unaffected by allogeneic PBMC

Cell monolayer formation and tightness were assessed on polyethersulfone membranes, which are already in use for hemodialysis treatments and were shown to be suitable for generation of ''living membranes'' with functional growth of PTEC [6,16-19]. CiPTEC-T1 were cultured on L-DOPA and collagen IV coated flat microPES membranes to grow stable cell monolayers, similarly to previously performed studies with microPES HFM [6,12]. Figure 4.6a shows the Transwell®-based culture system with microsPES membranes used to generate ciPTEC monolayers, and evaluate their stability after co-culture with PBMC by measuring inulin-FITC transepithelial diffusion. The ZO-1 expression confirms that ciPTEC-T1 form tight cell monolayers on microPES membranes (Figure 4.6b). Figure 4.6c shows inulin-FITC diffusion through untreated and cisplatin (50 μM) treated ciPTEC-T1 monolayer, indicating the specificity of barrier function. As shown in Figure 4.6d, inulin-FITC diffusion through double-coated microPES membranes carrying ciPTEC-T1 was 15 ± 1 $pmol \cdot min^{-1} \cdot cm^{-2}$, compared to 45 ± 2 $pmol \cdot min^{-1} \cdot cm^{-2}$ ($p<0.001$) and 44 ± 9 $pmol \cdot min^{-1} \cdot cm^{-2}$ ($p<0.01$) for non-coated membranes and double-coated membranes without cells, respectively. Importantly, co-culturing with PBMC did not alter the barrier function, even in the presence of strong PBMC stimulators such as LPS and anti-CD3/anti-CD28, or simultaneous exposure to plasma from ESRD patients (Figure 4.6d).

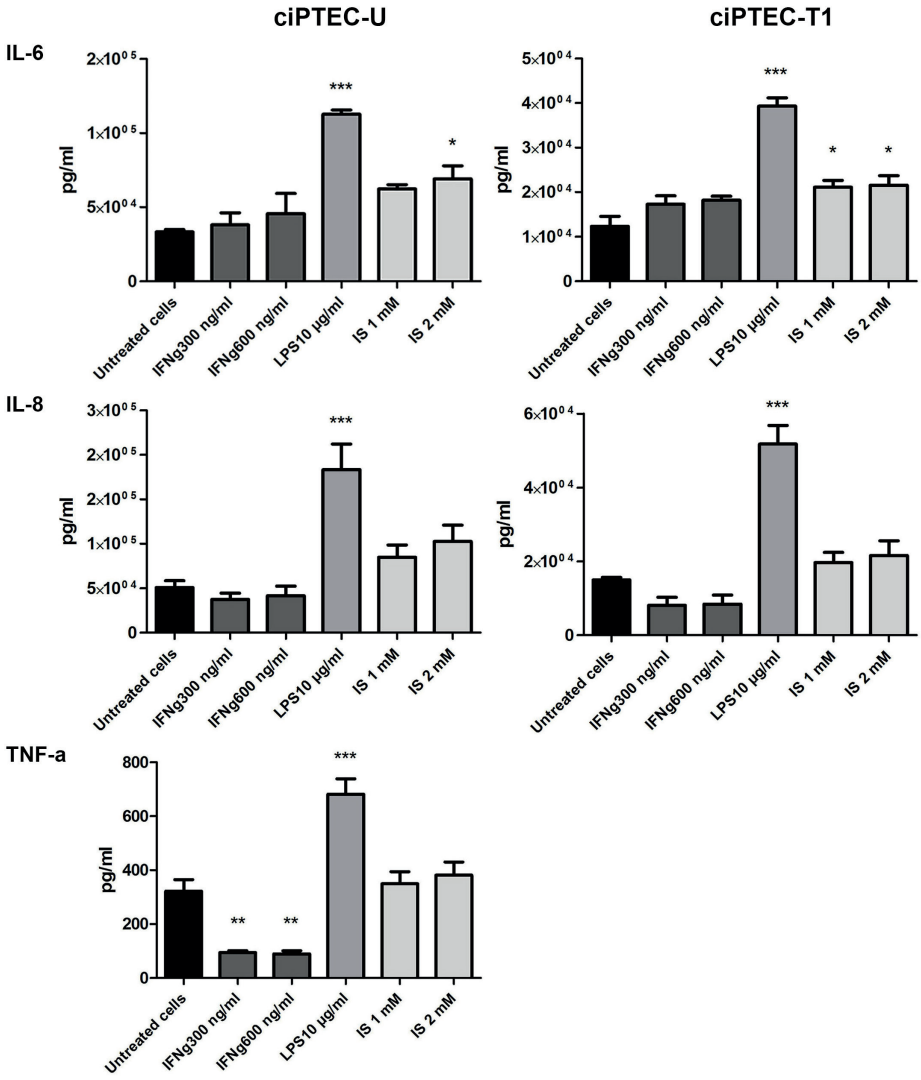


Figure 4.5. Proinflammatory cytokines production. Effect of 48 h exposure to IFN-γ (300 and 600 ng/ml), LPS (10 µg/ml) and indoxyl sulfate (IS) (1 and 2 mM) on proinflammatory cytokine production (IL-6, IL-8 and TNF-α) by ciPTEC-U and –T1. Concentration from three independent experiments is expressed as pg/ml (mean ± SEM). *p<0.05, **p<0.01, ***p<0.001, compared to untreated cells (One-way ANOVA, Dunnett's multiple comparison test).

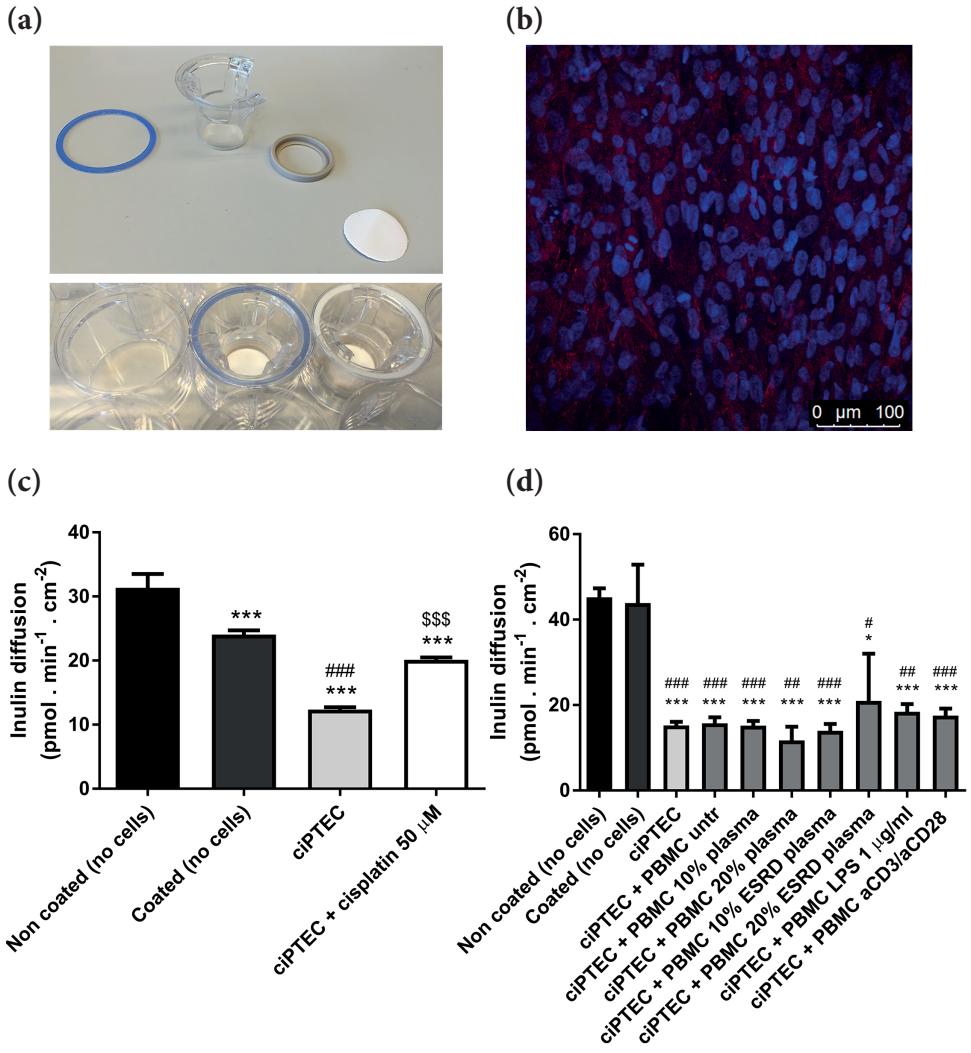


Figure 4.6. Epithelial cell monolayer formation and transepithelial barrier function following co-culture with PBMC. (**a**) Transwell® inserts and microPES hydrophilic flat membrane used for the indirect co-culture of ciPTEC-T1 and PBMC. (**b**) ZO-1 expression (red) in ciPTEC-T1 cultured on double-coated microPES membranes after 7 day maturation at 37°C, nuclei stained with DAPI (blue) (25X magnification). (**c**) Transepithelial inulin-FITC diffusion in absence of cells, in presence of double-coating (L-DOPA 2 mg/ml and collagen IV 25 µg/ml), untreated cells and cells exposed to cisplatin 50 µM for 24 h and (**d**) Iinulin-FITC diffusion across membranes containing ciPTEC-T1 after 48 h of co-culture with PBMC in the presence of several stimulatory conditions, including plasma from ESRD patients. Diffusion is shown as pmol·min^{-1}·cm^{-2} (mean ± SEM). Two independent experiments in triplicate for (**c**) and three independent experiments in duplicate for (**d**) were performed. *p<0.05, ***p<0.001, compared to non-coated membranes; # p<0.05, ## p<0.01 and ### p<0.001, compared to coated membranes; $$$ p<0.001, compared to ciPTEC (untreated control; One-way ANOVA, Dunnett's multiple comparison test).

DISCUSSION

In this study, we demonstrated the lack of immunogenic effects of ciPTEC studying several aspects of the immune system as a part of safety assessment required during the BAK device development. A prototype example of the BAK device and potential interactions with the host immune system is shown in Figure 4.7. When ciPTEC lines are used in a BAK, the cells will be derived from an unrelated individual. As other nucleated somatic cells [25], ciPTEC can be expected to express HLA class I antigens, which are highly polymorphic and able to induce alloreactive immune responses in cell, tissue, and organ transplantation. The expression of foreign HLA antigens on donor antigen presenting cells can directly activate host T cells, but the alloantigens can also be released in microvesicles or processed into peptides and semi-directly or indirectly presented by host APC to responding T cells [26]. Here, we demonstrate that our two ciPTEC lines, urine and kidney tissue derived, indeed express HLA class I molecules on their surface, and that this expression can be increased when the cells are exposed to IFN-γ and LPS. This is in line with other studies that have described an effect of IFN-γ on HLA expression in many cell types, including renal epithelial cells [27].

Besides the expression on cell membranes, soluble HLA molecules can be found in the circulation of healthy individuals [28]. High serum concentrations of sHLA class I molecules are related to a variety of diseases and clinical states, such as graft rejection, infection, cancer and autoimmune disease, in particular systemic lupus erythematosus and rheumatoid arthritis [29-31]. It has been described that sHLA can have immunomodulatory functions, mostly related to the induction and maintenance of self-tolerance [32,33]. The effect of sHLA in the setting of organ transplantation is still a matter of debate [34]. In case of a bioartificial device, it can be hypothesized that sHLA is adsorbed on the polymer fibers or is taken up by antigen presenting cells, which might mediate immunomodulation in patients via interaction with T cells [35,36]. We here showed that ciPTEC lines can release sHLA, especially after stimulation with IFN-γ as was described for other cell types [37] and in patients with cancer [38]. The concentration of sHLA in the culture supernatant was very low compared to serum concentrations of healthy individuals or kidney transplant recipients before and after transplantation [39,40] for instance, or patients with hemorrhagic fever with renal syndrome [41]. Nevertheless, the production levels of sHLA observed *in vitro* can still be different from more physyiological conditions of unidirectional flow and shear stress present in the BAK.

While HLA class I antigens are expressed on the surface of almost all nucleated cells, HLA class II proteins are found mostly on the surface of professional antigen presenting cells, endothelial cells, and activated T cells [42]. We found no expression of HLA-DR antigens on ciPTEC in untreated conditions and neither after exposure to several stimuli, except for the activated PBMC-CM. This is contrary to what was observed by Demmers *et al.*, who demonstrated HLA-DR expression on primary tubular epithelial cells in unstimulated conditions with an increase after IFN-γ stimulation [27]. We did not investigate further the levels of sHLA class II antigens, given the fact that HLA-DR is hardly expressed on ciPTEC and that the levels of sHLA class II proteins are usually low [43-45].

Additionally, we investigated the expression of the co-stimulatory molecules CD40, CD80, and CD86 to evaluate the capacity of ciPTEC to act as antigen presenting cells (APC). CD40 expression has been described for many cell types, such as lymphocytes, monocytes, dendritic cells, endothelial cells, smooth muscle cells, fibroblasts, as well as epithelial cells [46]. In line with previous studies [27,47], we showed that CD40 is expressed in both ciPTEC lines and its expression can be enhanced in the presence of activated PBMC-CM, indicating that the particular mixture of soluble factors present in the conditioned medium can have an effect on CD40 expression. This clearly implies that CD40, in an environment that resembles the activation of the adaptive immune system, can be upregulated and be involved in further amplification of the response. CD80 and CD86, also known as B7-1 and B7-2, respectively, are the two major types of the B7 family of membrane proteins which are usually expressed on APC and other myeloid cells and provide co-stimulatory signals in the interaction between APC and T cells, via binding to CD28. A previous study suggested that CD80 and CD86 can be expressed on PTEC under certain conditions when cells are exposed to several cytokines simultaneously, such as IFN-γ, IL-1α, IL-4, IL-13, in the presence of a CD40 ligand [48]. However, we did not observe any expression regardless the stimulation state, confirming the findings of Demmers *et al.* [27].

To evaluate the potential of ciPTEC to induce an alloimmune response, we performed direct co-culture experiments with ciPTEC and PBMC from healthy donors. This is the classical approach to measure the induction of cellular alloreactivity *in vitro* although the design of the BAK may not allow direct contact between ciPTEC and host immune cells (Figure 4.7). We showed that ciPTEC are not able to induce the activation and proliferation of PBMC, even if they were pre-stimulated under different conditions to enhance the expression of HLA antigens and co-stimulatory molecules and, therefore, the APC phenotype. The obtained data are in line with previous studies that showed that PBMC did not proliferate after five days of direct co-culture with renal tubular epithelial cells [49]. Assays for the measurement of indirect alloreactivity are difficult and have limited reproducibility [50]. Mostly, the response induced by the direct presentation of alloantigens is stronger than that induced by indirect presentation. Therefore, the absence of a response of PBMC after co-culture with ciPTEC lines underscores the safety of the BAK device.

As described previously, PTEC are able to secrete several proinflammatory cytokines [51,52]. We here confirmed that both ciPTEC lines were able to secrete IL-6 and IL-8, especially in response to LPS, which reflects an extreme inflammatory condition. TNF-α secretion was detected only in the supernatant of the urine derived ciPTEC line (ciPTEC-U) and was also strongly induced after stimulation with LPS. However, upon IFN-γ exposure the levels of TNF-α were significantly reduced, which can be due to the IFN-γ mediated downregulation of ADAM17, a metalloproteinase responsible for the cleavage of membrane-associated cytokines such as TNF-α [53] (Figure 4.S1). In the context of a BAK, the production of IL-6 and TNF-α might contribute to the activation of immune cells and propagation of inflammation usually present in CKD patients [54-57]. Moreover, IL-8 could promote the chemotaxis of inflammatory cells and their eventual adhesion to the fibers containing ciPTEC. Nevertheless, when cells are cultured on HFM in 3D

environment [16], there is a polarized, apical secretion of cytokines (N. Chevtchik, M. Mihajlovic *et al.,* unpublished data), suggesting that risks associated with inflammatory mediators are most likely minimal.

Besides characterizing ciPTEC and assessing their immunogenic potential, we also evaluated the effect of host immune cells on ciPTEC, resembling a possible BAK environment in which two cell populations are separated by a polymer membrane (Figure 4.7). Our previous studies, as well as those from other groups, showed that PTEC can form stable and tight monolayers [6,12,17,23,58,59]. In order to culture ciPTEC on microPES, a double coating given by L-DOPA and collagen IV is required [6,12]. In this study, we cultured ciPTEC-T1 on flat coated microPES membranes to investigate the integrity and barrier function of cell monolayer in (patho) physiologically relevant conditions for BAK. After formation of the monolayer, ciPTEC were exposed basolaterally to PBMC, in regular as well as stimulatory conditions. Neither inflammatory stimulation of PBMC, nor a uremic-like environment provided by pooled plasma from ESRD patients, did compromise the barrier function of ciPTEC monolayer as observed by inulin-FITC transepithelial diffusion. Cisplatin was able to alter monolayer integrity, as described previously [60-62], supporting the stability of barrier function. Such behavior of ciPTEC is very promising for their correct function in BAK.

Previously, ciPTEC-U and ciPTEC-T1 lines, were characterized and compared in a study by Jansen *et al.*, who showed that both types are functionally active, expressing several specific transporters while maintaining reabsorption mechanisms. The biggest difference encountered was related to a different profile of extracellular matrix (ECM) components produced by ciPTEC-U compared to ciPTEC-T1, which might explain the need for additional collagen IV coating to obtain tight monolayers [8]. In the present study, we investigated and characterized these two ciPTEC lines regarding their immunomodulatory functions and ability to act as non-professional APC. It has been suggested that epithelial cells can actually have the capacity to function as non-professional APC through the expression of HLA and co-stimulatory molecules [63]. We showed that there are no major differences between ciPTEC-U and ciPTEC-T1 with respect to the expression of HLA and co-stimulatory molecules, cytokine production and direct immunogenic effect, and neither were able to function as non-professional APC. Despite the fact that the final design of BAK is, as of yet, not completely established, the concept is to create a ciPTEC monolayer inside the HFM, for optimal function of BAK in terms of clearance. Considering that design, ciPTEC would be protected by a double-coated membrane from the blood compartment in the extracorporeal device, which would drastically, if not completely, reduce the risk of direct interaction with blood cells from the patient and immune system activation. Our data provide a first proof-of-concept encouraging further BAK development, though, a long road of research needs to be taken before such a concept can be applied in a clinical setting. Cell-engineered products, such as a BAK, need to obey guidelines as set for advanced therapy medicinal products (ATMPs) by the European Medicines Agency (EMA; Regulation EC No. 1394/2007) [64], and the U.S. Food and Drug Administration (FDA) [65], of which immune safety is one aspect. Other characteristics of intended cells, including the extent of replication competence of viruses, long

time functionality, ability to proliferate or differentiate, the risk of oncogenicity, *in vivo* efficacy, and mode of administration or application use, have to be determined. Experiments planned in the near future will address these issues.

In conclusion, ciPTEC appear to be a safe choice for BAK application, as far as it concerns their potential to induce an alloimmune response.

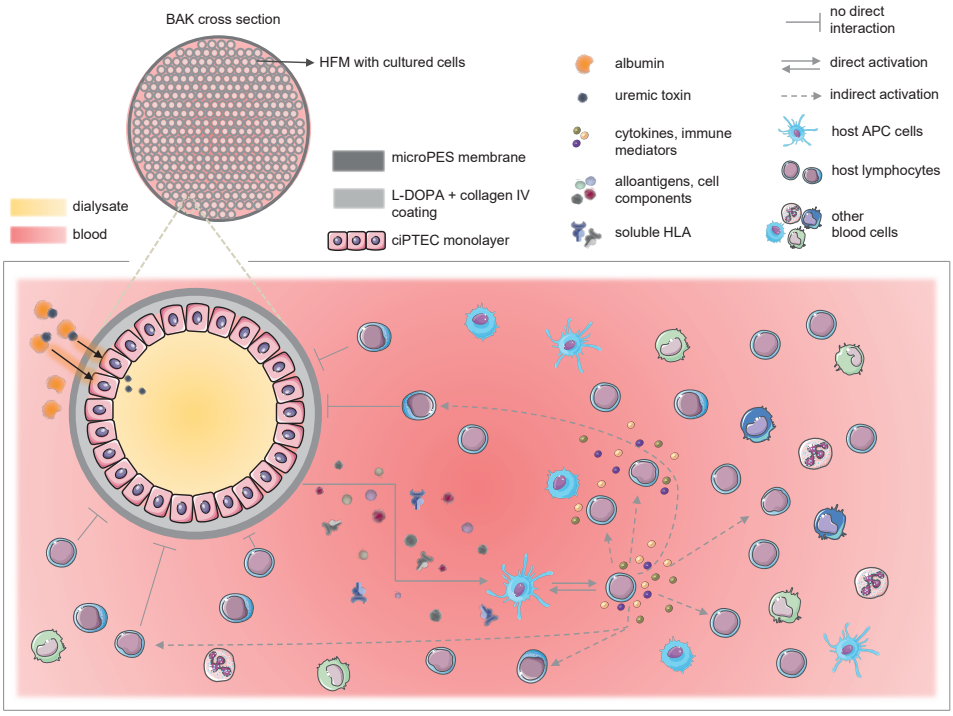


Figure 4.7. Schematic representation of BAK. The BAK device is composed of multiple HFM containing tight monolayers of ciPTEC for efficient removal of protein-bound uremic toxins. Uremic toxins bound to albumin can be taken up by ciPTEC from the blood side, and secreted into the dialysate compartment of the BAK. The presence of the polymer membrane should be sufficient to block direct interaction of host blood cells, in particular T cells, and ciPTEC via T-cell receptors and HLA molecules, thereby preventing direct activation of an immune response. However, there is a possibility of an immune response towards microvesicles, soluble alloantigens or other cellular components that might be released by ciPTEC. In that case, it can be expected that host APC could present ciPTEC-specific antigens to helper T cells, thus leading to a semi-direct or indirect pathway of immune response activation [26]. Once activated, helper T cells can cause the activation of other cells, such as cytotoxic T cells, B cells and macrophages, by releasing proinflammatory cytokines. This could potentially also lead to a non-specific inflammatory response, especially due to the excessive secretion of proinflammatory mediators by activated immune and inflammatory host cells.

ACKNOWLEDGMENTS

This research was supported by the Marie Curie ITN project BIOART (grant no. 316690, EU-FP7-PEOPLE-ITN-2012). We would like to thank Gemma Dingjan for technical support with flow cytometry. Also, we are grateful to Carolien Schophuizen for the support with custom-made Transwell® culture systems, Elena Kamburova for her help with co-culture experiments and Diënty Hazenbrink for providing us with human plasma samples.

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SUPPLEMENTARY INFORMATION

Table 4.S1. PCR-SSO typing of HLA isotypes of two ciPTEC cell lines. The HLA-A, -B and –C alleles were examined in MHC class-I locus and HLA-DRB, -DQA and –DQB in MHC class-II locus.

	HLA-I			HLA-II		
	HLA-A	**HLA-B**	**HLA-C**	**HLA-DRB**	**HLA-DQA**	**HLA-DQB**
ciPTEC-U	A*01 A*24	B*07 B*08	C*07:01 C*07:02	DRB1*03 DRB1*15	DQA1*01 DQA1*05:01	DQB1*02 DQB1*06
ciPTEC-T1	A*02 A*03	B*07 B*40:01	C*03:04 C*07:02	DRB1*11 DRB1*13	DQA1*01 DQA1*05	DQB1*03:01 DQB1*06

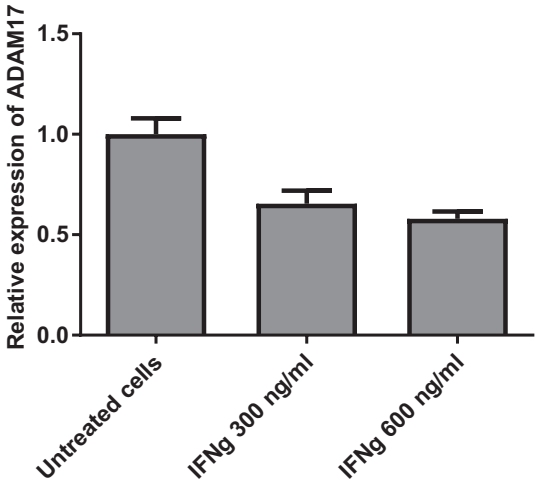

Figure 4.S1. Relative gene expression of ADAM17 in ciPTEC-U. Gene expression was assessed after 48 h exposure to IFN-γ (300 and 600 ng/ml). GAPDH used as a house-keeping reference gene. Experiment was performed once in duplicates. Specific sense and anti-sense primers for ADAM17 (forward: TCCAGCAGCATTCGGTAAGAA; reverse: AGAGTCAGGCTCACCAACCA) and GAPDH (forward: ACAGTCAGCCGCATCTTCTT; reverse: ACGACCAAATCCGTTGACTC) were synthesized by Biolegio (Nijmegen, The Netherlands). Gene expression was performed by total RNA isolation using RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions, followed by cDNA synthesis using the Omniscript RT-kit (Qiagen, Venlo, the Netherlands) and Real-Time PCR using the iQ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The data were analyzed using Bio-Rad CFX Manager™ Software version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA).

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Accepted for publication in Journal of Tissue Engineering and Regenerative Medicine
doi: 10.1002/term.2694

CHAPTER V

A BIOARTIFICIAL KIDNEY DEVICE WITH POLARIZED SECRETION OF IMMUNE MODULATORS

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ABSTRACT

The accumulation of protein-bound toxins in dialyzed patients is strongly associated with their high morbidity and mortality. The bioartificial kidney device (BAK), containing proximal tubule epithelial cells (PTEC) seeded on functionalized synthetic hollow fiber membranes (HFM), may be a powerful solution for the active removal of those metabolites. In an earlier study, we developed an upscaled BAK containing conditionally immortalized human PTEC (ciPTEC) with functional organic cationic transporter 2 (OCT2). Here, we first extended this development to a BAK device having cells with the organic anionic transporter 1 (OAT1), capable of removing anionic uremic wastes. We confirmed the quality of the ciPTEC monolayer by confocal microscopy and paracellular inulin-FITC leakage, as well as, by the active transport of anionic toxin, indoxyl sulfate (IS). Furthermore, we assessed the immune-safety of our system by measuring the production of relevant cytokines by the cells after lipopolysaccharide (LPS) stimulation. Upon LPS treatment, we observed a polarized secretion of proinflammatory cytokines by the cells: 10-fold higher in the extraluminal space, corresponding to the urine compartment, as compared to the intraluminal space, corresponding to the blood compartment. To the best of our knowledge, our work is the first to show this favorable cell polarization in a BAK upscaled device.

Keywords: bioartificial kidney; living membrane; ciPTEC monolayer; organic anionic transporter; polarized secretion of immune modulators

INTRODUCTION

Despite the ongoing progress in dialysis therapy, only small and middle-size xenobiotics can be eliminated. The removal of bigger size solutes and protein-bound toxins is limited [1,2]. Recently, the accumulation of these protein-bound solutes has been strongly associated with the fatal outcome of the patients [3,4]. Therefore, there is a strong need for novel strategies and concepts for their removal [5], such as a bioartificial kidney (BAK). The BAK aims at mimicking the functional kidney by making use of proximal tubule epithelial cells (PTEC), equipped with a broad range of transporters, which normally mediate the excretion of those solutes [6]. This device consists of living membranes containing tight monolayer of renal cells with preserved functional organic ion transporters, grown on an artificial porous hollow fiber membrane (HFM).

In recent years, several studies have presented BAK prototypes making use of human PTEC [7-14], showing a preserved phenotype and sometimes metabolic and/or endocrine functions *in vitro* or *in vivo*. However, mostly primary cells were used which are characterized by limited availability, donor to donor variation and the loss of phenotype or functionality upon culturing. The recently developed and well-characterized human conditionally immortalized PTEC (ciPTEC) line appears to be a suitable candidate for an efficient BAK system [15-21]. These cells are transduced with human telomerase (hTERT) that limits replicative senescence by telomere length maintenance. In addition, their proliferation is controlled by the temperature sensitive

mutant of SV40 Large T antigen (U19tsA58), allowing proliferation at 33 °C and differentiation in mature PTEC at 37 °C. Due to these modifications the cell line has high availability, limited senescence, and can be used up to a high passage number.

A recent study on small single HFM showed an active excretion of IS and kynurenic acid (KA) by ciPTEC [18] through the concerted action of organic anion transporter-1 (OAT1), breast cancer resistance protein (BCRP) and multidrug resistance protein-4 (MRP4). This property of the ciPTEC is of high importance for BAK application, considering that most of the protein-bound toxins are anionic molecules [22,23]. In the present work, we first developed an upscaled living membrane to support the OAT1-expressing ciPTEC line. The transport properties and the quality and function of the grown ciPTEC monolayer were investigated, including the expression of zonula occludens-1 (ZO-1) protein and the diffusion of fluorescein isothiocyanate (FITC)-labelled-inulin (inulin-FITC). Furthermore, we studied the transport of an anionic uremic toxin, IS, mediated by the combined action of OAT1, BRCP and MRP4, in the absence or in the presence of the OAT1 inhibitor, probenecid.

A very important issue related to the clinical implementation of the BAK device is its safety. It is crucial to investigate whether the device with the human allogenic cells induces immune and inflammatory responses in the host. Besides, the high uremic toxins concentrations in kidney patients are often associated with inflammation, which may as well be detrimental for the BAK [24]. A first assessment of the immunogenicity of the ciPTEC lines using flat membranes with a small surface area (1.12 cm^2) showed that ciPTEC have low immunogenicity *in vitro*, although able to secrete several proinflammatory cytokines that could potentially mediate a non-specific inflammatory response [25]. In this work, we investigated whether there is polarization of the production of proinflammatory and immune mediators by the ciPTEC cultured in the BAK system. We measured the release of the most relevant proinflammatory mediators – IL-6, IL-8 and TNF-α - and sHLA–class I without or with exposure to lipopolysaccharide (LPS) or interferon-γ (IFN-γ) in both – dialysate and blood – compartments of the system. To the best of our knowledge, our work is the first to focus on this important issue for the development of BAK devices.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. MicroPES TF10 hollow fiber capillary membranes (HFM) (wall thickness 100 μm, inner diameter 300 μm, max pore size 0.5 μm) were purchased from 3M - Membrana GmbH (Wuppertal, Germany).

Module preparation, HFM sterilization, coating and characterization

The modules were prepared following the protocol presented previously [20]. A dual coating of L-DOPA and Collagen IV coating was applied to the fibers, at 37 °C on a shaking device, for a duration of 20 h and 2 h respectively [20]. The HFM transport properties (water permeability) was measured before and after sterilization and before and after coatings, in the absence of ciPTEC. An OSMO Inspector automated setup (Convergence B.V, Enschede, The Netherlands) was used to quantify the clean water (Merck Millipore, Billerica, MA) flux (CWF) through the HFM (J, in $l/(m^2 \cdot h^1)$) as a function of the transmembrane pressure (TMP or ΔP, in bar). The membrane permeance (L, in $l/(m^2 \cdot h^1 \cdot bar^1)$) was calculated from the slope of the curve. Every pressure step was maintained for 30 min.

Cell culture and modules handling

The ciPTEC OAT-1 expressing, urine-derived, cell line [15,21] was cultured at proliferating temperature of 33°C and maturation temperature of 37°C in ciPTEC complete medium. The latter was prepared as described previously [20] with the difference that ciPTEC were always cultured in absence of antibiotics up to a maximum of 60 passages. The modules' handling and cell seeding was performed as reported previously [20]. Briefly, prior to cell seeding, modules were incubated for 1 h in ciPTEC complete medium. Proliferating 90% confluent ciPTEC were detached using Accutase (StemPro® Accutase®, Life Technologies Europe BV, Bleiswijk, the Netherlands), centrifuged and suspended at a concentration of 2.0-2.5 million cells/ml in the ciPTEC complete medium. The modules' extraluminal space was completely filled with the cell suspension. To promote initial cell attachment, the modules were placed at 33°C, 5% CO_2 for 8 h, with a rotation of 90 degrees every 2 h. Afterwards, the modules were washed with the ciPTEC complete medium, provided with gas exchange filters and the cell proliferation was allowed for additional 64 h. Finally, the temperature was changed to 37°C for 7 days to allow ciPTEC maturation. During the culture period, ciPTEC were supplemented with fresh culture medium every second day.

Immunocytochemistry

Matured ciPTEC were fixed using 2 w/v% paraformaldehyde in HBSS supplemented with 2 w/v% sucrose for 10 min and permeabilized in 0.3 v/v% triton X-100 in HBSS for 10 min. To prevent non-specific binding of antibodies, cells were exposed to block solution containing 2 w/v% bovine serum albumin, 2 v/v% fetal calf serum and 0.1 v/v% Tween-20 in HBSS for 30 min. Cells were incubated with antibodies against the tight junction protein zonula occludens 1 (ZO-1, 1:50 dilution in block solution, Invitrogen, Carlsbad, CA) for 90 min, followed by a simultaneous incubation with goat-anti-rabbit-Alexa 568 conjugate (1:200, Life Technologies Europe BV, Bleiswijk, The Netherlands) and Phalloidin-Atto 488 (1:500), for actin filaments staining, for 30 min. Finally, DAPI nuclei staining (300 nM, Life Technologies Europe BV) was performed for 5 min. The modules were carefully cut open and the extracted fibers were mounted on microscopy slides using Dako fluorescent mounting media (Dako Netherlands B.V, Heverlee, Belgium). The

slides were examined under the Nikon confocal A1/ super resolution N-STORM microscope (Nikon Instruments Europe B.V, Amsterdam, The Netherlands). Images were captured using the NIS-elements analysis software, version 4.40.000.

Transepithelial barrier function

Paracellular permeability of the mature living membranes was quantified following the previously described method [20]. After washing the modules with Krebs-Henseleit buffer supplemented with HEPES (10 mM; KHH buffer), inulin-FITC (0.1 mg/ml in KHH buffer) was perfused at 18 ml/h at 37°C for 15 min. The inulin-FITC leakage was determined prior to perform functional IS transport. In some of the modules, the inulin-FITC leakage was determined before and after the IS transport experiment and after LPS or IFN-γ exposure to assess the integrity of the monolayer after the functional tests.

Functional organic anion transport

Transepithelial transport of IS through the HFM with matured ciPTEC was studied using a similar perfusion set up as the one used for the barrier function assay. First, fibers were pre-incubated in KHH buffer without or with probenecid (p) at concentrations of 100 μM (p100) or 500 μM (p500) at 37°C for 15 min. Next, the fibers were perfused using 100 μM IS in KHH buffer in the presence or absence of inhibitors for 10 min at a flow rate of 18 ml/h. Samples from both permeate and outlet were collected. IS concentrations were measured with a Jasco HPLC system equipped with a pump (PU-2080) an autosampler (PF-2020), UV/VIS detector (UV-2070), fluorescence detector (FP-2020). The protocol was adapted from [26]. The excitation and emission wavelength for the fluorescence detector were set at 272 nm and 374 nm, respectively. The eluents used were ammonium formate (MW 63.06 g/mol) buffer 50mM + 10% methanol at pH = 3.0. The flow rate was set at 0.5 ml/min.

Production of the proinflammatory cytokines and human leukocyte antigens

The apical and basolateral sides of the living membrane were exposed to the following treatments depending on the experimental set up: interferon-γ (IFN-γ) 300 ng/ml, lipopolysaccharide (LPS) 10 μg/ml. The production of IL-6, IL-8, TNF-α and soluble HLA-class I (sHLA-class I) molecules in various stimulatory conditions, was measured by means of enzyme-linked immunosorbent assays (ELISAs). Cell culture supernatants were collected from apical (external, volume approximately 3 ml) and basolateral (internal, volume approximately 0.4 ml) compartments after exposure to various stimulatory conditions, centrifuged for 10 min at 240 g, 4°C, and stored at -80°C. DuoSet® ELISA Development Systems kits (IL-6 #DY206, TNF-α #DY210, IL-8 #DY208; R&D systems, Abingdon, UK) and Human MHC class-I kit (Proteintech, Chicago, IL, USA) were used to quantify the cytokine and sHLA-class I levels respectively, in cell culture supernatants according to manufacturer's instructions. The optical density was determined immediately using the iMark Microplate Absorbance Reader (Bio-Rad, Japan) set to 450 nm. Each sample was

measured in duplicates and quantification was done using Microplate Manager Software (version 6.0, Bio-Rad Laboratories, Hercules, CA, USA) capable of generating a four-parameter logistic (4-PL) curve-fit.

Data analysis

Every experiment was performed at least in duplicate. The number of samples (n) measured is indicated in each figure legend. The results are presented as mean ± standard deviation for inulin leakage and IS transport or standard error of the mean for concentration of immune modulators. Statistical analysis of the cell monolayer integrity and function was performed in the SPSS software (IBPM SPSS Statistics version 23.0) using one-way analysis of variance (ANOVA) or Student's t-test, where appropriate. Statistical analysis of the production of proinflammatory cytokines was performed in the GraphPad Prism software (GraphPad software, version 5.03; La Jolla, CA, USA), using either unpaired two-tailed test (for differences between two compartments in the same module) or one-way ANOVA followed by Tukey's multiple comparison test (for differences between treatments and different modules). A p-value < 0.05 was considered significantly different.

RESULTS

HFM characterization

The HFM were sterilized with steam to prevent risks of infection and coated with L-DOPA and collagen IV to allow ciPTEC adhesion and function. Prior to performing cell culturing experiments the potential impact of sterilization and coating on the membrane transport properties were evaluated. Figure 5.1 compares the clean water flux (CWF) through the unsterilized or sterilized, and uncoated or coated HFM at different pressures. The steam sterilization did not affect the permeance of the uncoated membranes ($(19.2 \pm 0.9) \cdot 10^3$ l/($m^2 \cdot h^1 \cdot bar^1$)) unsterilized versus ($18.9 \pm 0.8) \cdot 10^3$ l/($m^2 \cdot h^1 \cdot bar^1$)) sterilized). The permeance was slightly decreased when the double coating was applied on the sterilized HFM ($(16.9 \pm 0.7) \cdot 10^3$ l/($m^2 \cdot h^1 \cdot bar^1$)) compared to the non-sterilized HFM ($(19.0 \pm 0.3) \cdot 10^3$ l/($m^2 \cdot h^1 \cdot bar^1$)), but remains high. This may be due to increased wettability of the membranes after sterilization, which favors the coating deposition and results in a slight pore occlusion. The SEM analysis shows no difference between the surfaces and cross-sections of the uncoated and coated HFM (Figures 5.S1 and 5.S2). Overall, these results indicate that the high HFM permeance is preserved even after the sterilization and coating procedures.

Cell monolayer integrity and function

Figure 5.2a-b shows representative images of matured OAT1-expressing ciPTEC cultured on MicroPES HFM. The applied L-DOPA (20 h) and collagen IV (2 h) coating supports the formation of a uniform ciPTEC monolayer. The abundant expression of the Zonula Occludens 1 (ZO-1) (Figure 5.2b) is demonstrative of the presence of tight junctions between the cells. In addition to a polarized epithelial barrier, the tight junction proteins contribute to fluid and

ion homeostasis mediated by paracellular transport [27]. Besides, cytoskeleton (actin) staining (Figure 5.2a) shows that the cells have proper shape, structure and morphology, additionally confirming the epithelial character of the cell monolayer.

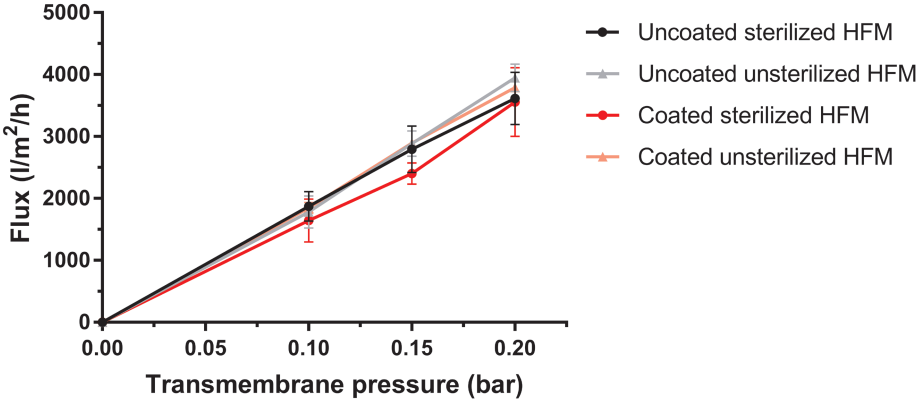

Figure 5.1. HFM permeability. Clean water fluxes (CWF) for unsterilized and sterilized HFM, with and without coating. The slope of the CWF as a function of the pressure gives the HFM permeance. Data are shown as mean ± standard deviation of three or four samples.

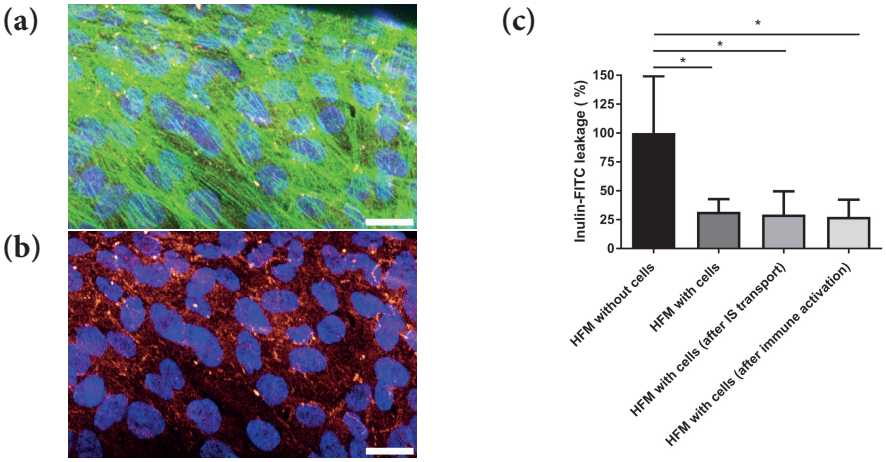

Figure 5.2. Monolayer quality of ciPTEC cultured on HFM. (**a**, **b**) Representative confocal microscopy images of ciPTEC cultured on HFM; DAPI-stained nuclei (blue), the cytoskeleton (green) and the ZO-1 (red). (**c**) Inulin-FITC paracellular leakage, ratio HFM with cells/HFM without cells. The data presented here arise from 4 experiments and 32 different modules. Data are presented as mean ± standard deviation. * $p < 0.001$ (one-way ANOVA).

Inulin transport through the mature cell monolayer occurs in a non-active manner, via diffusion through the inter-cellular junctions. Therefore, the inulin-FITC leakage test is representative of the monolayer tightness [17]. For a tight cell monolayer, inulin-FITC leakage is expected to be considerably lower than through HFM without cells. Figure 5.2c shows the inulin-FITC leakage through tested HFM, for which the fibers without cells are set to 100 %. The leakage of the HFM with a ciPTEC monolayer is as low as 32 ± 11 %.

We next assessed the activity of the transporter responsible for anionic uremic toxin excretion, OAT1, by perfusing the living membranes with IS in the absence or presence of the OAT1 inhibitor, probenecid. Figure 5.3a shows the scheme of the experimental set up and Figure 5.3b shows the transport results where the IS transport alone through the cell monolayer was set at 100 %. The IS transport is inhibited by approximately 50 % by both concentrations of probenecid (IS transport becomes 48 ± 20 % and 50 ± 15 % of the original intensity, for p100 and p500 respectively), suggesting maintained cell function. The inhibition of IS transport was comparable for both concentrations of probenecid, suggesting that the saturation of the transporters was reached. After the IS transport measurements, the cell monolayer was still intact as suggested by the low inulin-FITC leakage of around 30 % (Figure 5.2c).

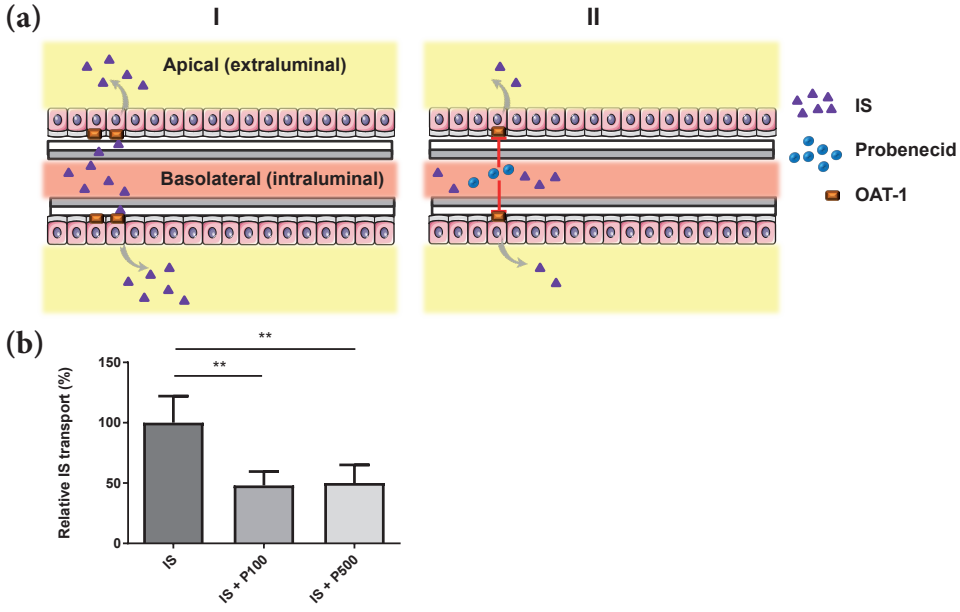

Figure 5.3. Functional OAT1-mediated IS transport. (**a**) Schematic presentation of the experimental set up of IS transepithelial transport in the absence (I) or presence (II) of probenecid. (**b**) Quantification of IS transport (100 µM) in the absence or presence of probenecid (concentration 100 µM –p100 and 500 µM – p500) in matured ciPTEC cultured on upscaled HFM. The perfusion lasted for 10 min. Data are normalized against IS transport in the absence of inhibitors and presented as mean ± standard deviation of at least 3 modules per case, from two independent experiments. *p < 0.001 (one-way ANOVA).

Production of the inflammatory and immune mediators

To assess whether the living membrane mediates an inflammatory or immune response, we evaluated the release of IL-6, IL-8, TNF-α and sHLA-class I in both extraluminal (apical) and intraluminal (basolateral) compartments. Figure 5.4a shows a schematic representation of the experimental set up in the case of exposure to LPS; an identical set up was used for the exposure to IFN-γ and the release of sHLA-class I. Figure 5.4b-e shows the release of proinflammatory cytokines IL-6, IL-8 and TNF-α and the sHLA-class I.

Prior to exposure to LPS, the concentrations of IL-6 and IL-8 were found to be 2 to 3 times higher in the extraluminal compartment ($(27 \pm 4) \cdot 10^3$ pg/ml and $(10 \pm 1) \cdot 10^3$ pg/ml respectively) than in the intraluminal compartment ($(9 \pm 4) \cdot 10^3$ pg/ml and $(4 \pm 1) \cdot 10^3$ pg/ml respectively). When the ciPTEC were directly exposed to LPS, the concentration of IL-6 and IL-8 increased in the extraluminal compartment (more than 10 times – $(496 \pm 267) \cdot 10^3$ pg/ml and $(103 \pm 9) \cdot 10^3$ pg/ml respectively). When LPS was administrated intraluminally, the extraluminal concentration of IL-6 and IL-8 increased slightly less (more than 5 times – $(100 \pm 15) \cdot 10^3$ pg/ml and $(67 \pm 7) \cdot 10^3$ pg/ml, respectively), most likely due to the small mass transfer resistance of the HFM. On the other hand, the intraluminal concentration of IL-6 remained the same for both extraluminal and intraluminal exposures to LPS ($(19 \pm 4) \cdot 10^3$ pg/ml and $(16 \pm 2) \cdot 10^3$ pg/ml, respectively). In similar conditions, the intraluminal concentration of IL-8 slightly increased: 2 times after extraluminal exposure and 3 times after intraluminal exposure ($(9 \pm 2) \cdot 10^3$ pg/ml and $(13 \pm 2) \cdot 10^3$ pg/ml, respectively).

The concentrations of TNF-α did not vary in all cases and remained very low (100-300 pg/mL), in agreement with our previous findings [25,28]. If we, however, estimate the absolute amount of TNF-α in both compartments (based on the volume of each compartment, we observe that the amount of TNF-α is higher in the extraluminal than in the intraluminal compartment) (Figure 5.S3d).

Similarly to TNF-α, the absolute levels of sHLA-class I were rather low (concentrations < 20 pg/ml) compared to normal serum levels [29,30]. Hence, in agreement with IL-6 and IL-8, the concentrations of sHLA-class I were 2 to 3 times higher in the apical than in the basolateral compartment (15 ± 6 pg/ml and 5 ± 1 pg/ml respectively). The apical concentration of sHLA-class I did not significantly change after the apical and basolateral exposures to IFN-γ (20 ± 5 pg/ml and 19 ± 4 pg/ml respectively), although a slight (2-fold) increase was observed in the basolateral compartment after basolateral exposure (9 ± 2 pg/ml).

Overall, for all of the configurations tested, the concentration of IL-6, IL-8 and sHLA-class I was higher in the extraluminal compartment, corresponding to dialysate, compared to the intraluminal one, which would correspond to patient's blood. This conclusion holds true also for the absolute amounts of proinflammatory cytokines and sHLA-class I in both compartments (Figure 5.S3). Finally, to assess the integrity of the cell monolayer during these tests, additional Inulin-FITC leakage test was performed on the HFM after exposure to stimulatory conditions (LPS and IFN-γ), which was found to be rather low, 27.4 ± 14.9 % (Figure 5.2c), and similar to that before the stimulations.

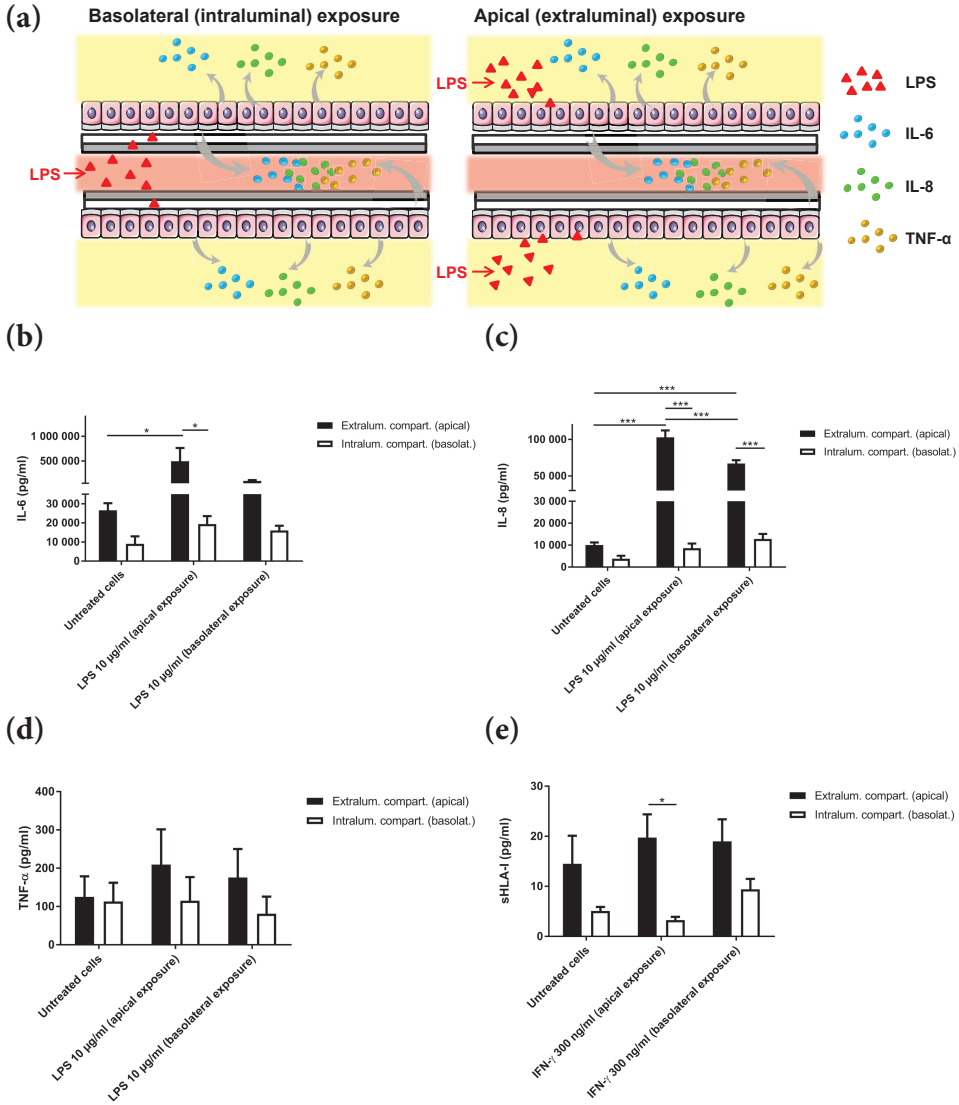

Figure 5.4. Production of proinflammatory cytokines and sHLA-class I by matured ciPTEC in upscaled BAK. (**a**) Scheme of the experimental set up used for studying the release of the proinflammatory cytokines, here after exposure to LPS and measure of IL-6, IL-8 and TNF-α; an identical setup was used in case of IFN-γ treatment and sHLA-class I release. (**b**, **c**, **d**, **e**) Effect of 24 h basolateral or apical exposure to LPS (10 µg/ml) (**b**, **c**, **d**) or IFN-γ (300 ng/ml) (**e**) on production of proinflammatory cytokines (IL-6, IL-8 and TNF-α) and sHLA-class I in the apical and basolateral compartments. Data are presented as mean ± standard error mean (SEM) of at least 3 modules per case, from two independent experiments. *p < 0.05 and ***p < 0.001 using either unpaired two-tailed test (for differences between two compartments in the same module) or one-way ANOVA followed by Tukey's multiple comparison test (for differences between treatments and different modules).

DISCUSSION

In this study, we developed upscaled (4 cm^2) 3D modules containing double coated MicroPES HFM supporting OAT1-expressing ciPTEC. With this system, we investigated the secretion of the proinflammatory cytokines IL-6 and IL-8 and sHLA-class by the cells in response to LPS or IFN-γ, both basolaterally and apically, to mimic inflammatory conditions.

Confocal microscopic analysis of the ciPTEC monolayer after 7 days of maturation at 37°C demonstrated regular nuclei, homogeneous cell structure and morphology, and abundant expression of the tight junction protein ZO-1. This underlines the epithelial character of the ciPTEC monolayer, which was confirmed further by limited inulin-FITC diffusion in agreement with earlier studies. Indeed, the earlier reported values - 30 ± 10 % for an upscaled system [20] and 31 ± 9 % [19] for a small scale system – are identical to the Inulin-FITC leakage percentage - 32 ± 11 % - reported here. In addition, the paracellular inulin-FITC leakage remained low after exposure to IS, LPS or IFN-γ, confirming the preservation of intact ciPTEC monolayers after the exposure to toxins and to inflammatory stimuli.

Our system demonstrated the active transport of IS, an anionic uremic toxin from the family of the protein bound toxins [23], reported as strongly linked to the fatal outcome in kidney patients [4,31]. This active transport indicates the presence of functional OAT1-mediated transport. OAT1 is expressed at the basolateral membrane of PTEC, responsible for the uptake of anionic uremic metabolites [18,21] and is crucial for their renal elimination. BCRP and MRP4 transporters, located on the apical membrane of PTEC, are responsible for the toxin excretion to the urine in the matured ciPTEC monolayer [18]. When the modules were incubated with probenecid, an inhibitor of OAT1, the transport of IS was reduced by approximatively 40%. In comparison to the study performed on a short single HFM [18], we also found a similar inhibition of the transport of IS (100 μM) by probenecid 100 μM: 48 ± 31 % [18] in the single small HFM versus 55 ± 6 % now in the upscaled system, $p < 0.001$.

Finally, we studied the production of IL-6, IL-8, TNF-α and sHLA-class I, in basic conditions and in response to LPS and IFN-γ, which we used to mimic inflammatory conditions as observed in uremic syndrome. For the BAK, the production of proinflammatory cytokines is important for the activation of immune cells and propagation of inflammatory response usually present in CKD patients, which could have undesirable effects. In most studies on BAK reported in the literature [7-9,13], the concentrations of cytokines were measured only from the waste (apical) compartment *in vitro* or the blood (basolateral) compartment *in vivo*. In our work, we measured the concentrations of proinflammatory cytokines in both apical and basolateral compartments and we found that they were very low and that their absolute values between extraluminal and basolateral compartment were significantly different. Both in the absence and in the presence of LPS or IFN-γ, the secretion of IL-6, IL-8 and of sHLA-class I was significantly higher to the apical side - corresponding to the dialysate compartment - compared to the basolateral secretion - corresponding to the blood side.

Overall, our results indicate that the BAK device described here consists of functional polarized ciPTEC, which secrete higher amounts of cytokines and sHLA-class-I molecules towards the dialysate compartment, thereby greatly reducing the risks associated with eventual proinflammatory and immunogenic effects of the cells.

CONCLUSION & OUTLOOK

This work presents the successful upscaling of the living membranes containing functional OAT1-mediated transport crucial for the removal of uremic anionic toxins, such as IS. Importantly, the ciPTEC were fully polarized since the release of proinflammatory cytokines IL-6 and IL-8 and of sHLA-class I molecules was mainly oriented towards the apical side, or dialysate compartment, and not towards the basolateral side – corresponding to the side of the patient's body fluid.

The next step towards the development of a functional BAK device is to culture the cells while exposing them to a unidirectional flow with relative shear stress to mimic the natural kidney proximal tubule physiology. There is evidence that cell metabolism is stimulated when cells are cultured under dynamic conditions [32-36], which might further stimulate toxin removal. Moreover, a ciPTEC-based BAK device should ensure a sufficient toxin clearance for prolonged sessions. Here, the function of the living membrane was tested for 10 min. Future work should evaluate longer clearance periods, using plasma or blood samples from CKD patients.

ACKNOWLEDGMENTS

This work was funded by the EU Marie Curie ITN Project BIOART (grant no.316690, EU-FP7-PEOPLE-ITN-2012). The authors would like to gratefully thank the EuTox Working Group, of the European society for artificial organs for its financial contribution. Natalia V. Chevtchik would like to gratefully thank the BioNanoLab of the University of Twente, the Netherlands for its financial contribution. Igor Middel of Utrecht University is acknowledged for his help in IS measurements. The authors would like to gratefully thank Felix Broens and Convergence Industry B.V. for the technical assistance in the program development for membrane properties characterization.

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SUPPLEMENTARY INFORMATION

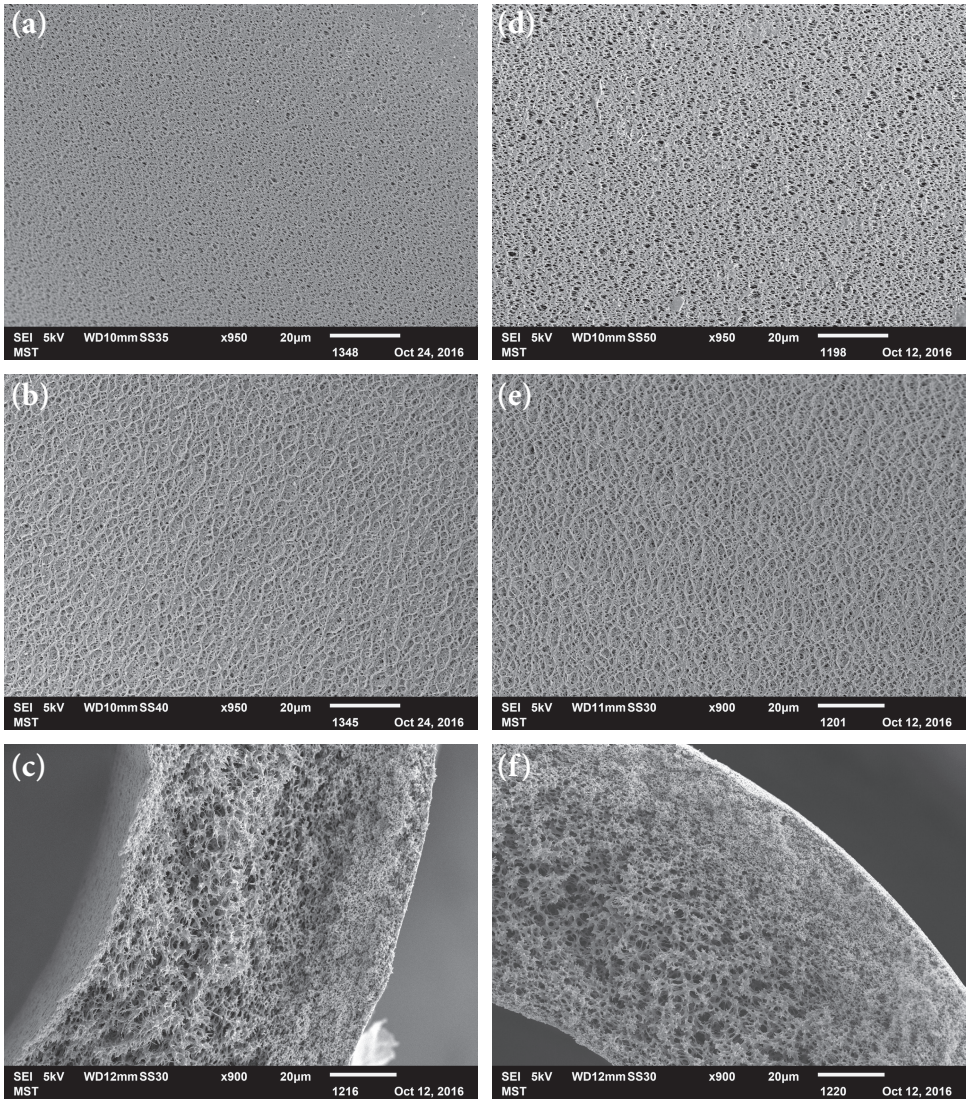

Figure 5.S1. SEM images of sterilized uncoated and coated MicroPES HFM. Magnification x900 and x950. (**a**, **b**, **c**) uncoated and (**d**, **e**, **f**) with L-DOPA and collagen IV double coating. HFM (**a**, **d**) outside surface, (**b**, **e**) inside surface and (**c**, **f**) cross-section.

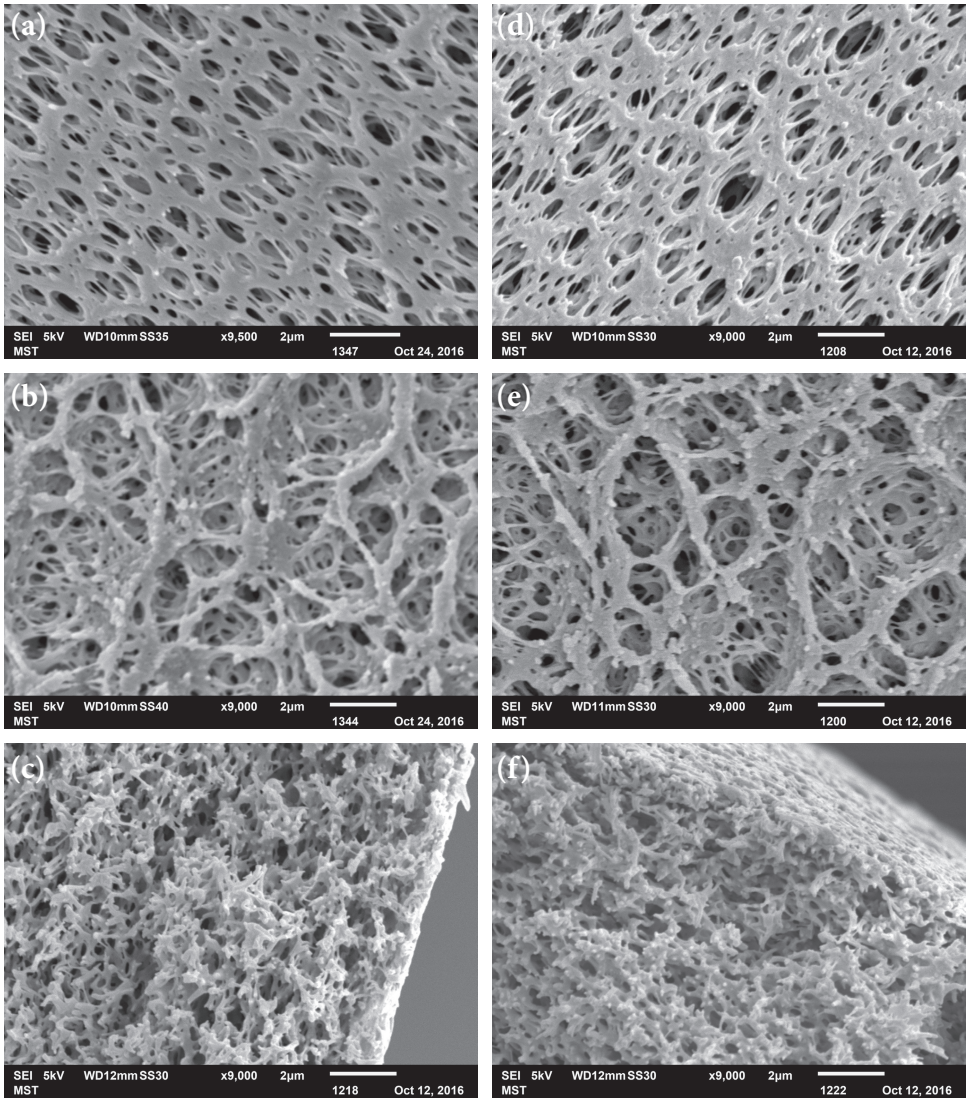

Figure 5.S2. SEM images of sterilized uncoated and coated MicroPES HFM. Magnification x9000. (**a**, **b**, **c**) uncoated and (**d**, **e**, **f**) with L-DOPA and collagen IV double coating. HFM (**a**, **d**) outside surface, (**b**, **e**) inside surface and (**c**, **f**) cross-section.

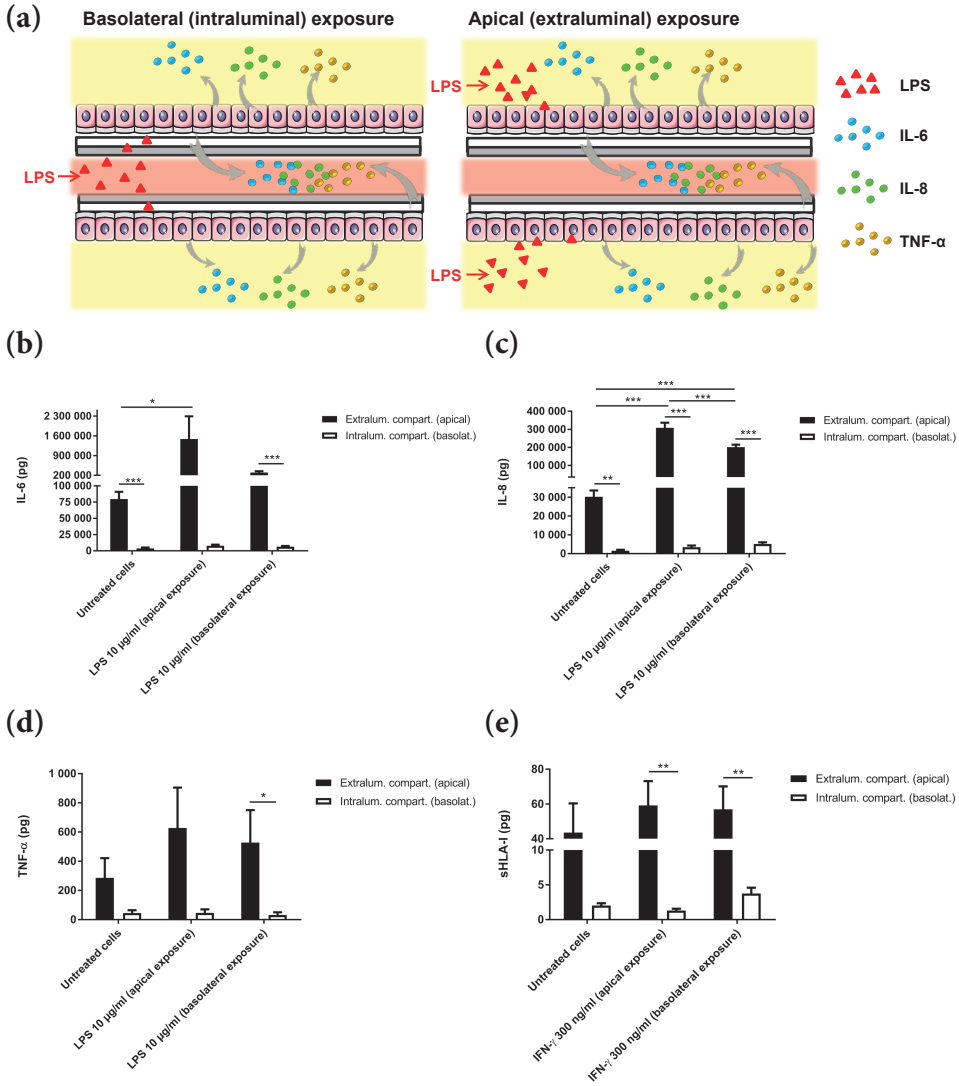

Figure 5.S3. Absolute values of produced proinflammatory cytokines by matured ciPTEC in upscaled BAK. (**a**) Scheme of the experimental set up used for the study of the release of the proinflammatory cytokines, here after exposure to LPS and measure of IL-6, IL-8 and TNF-α; an identical set up was used in case of IFN-γ treatment and sHLA-class I release. (**b**, **c**, **d**, **e**) Effect of 24 h basolateral or apical exposure to LPS (10 µg/ml) (**b**, **c**, **d**) or IFN-γ (300 ng/ml) (**e**) on production of proinflammatory cytokines (IL-6, IL-8 and TNF-α) and sHLA-class I in the apical and basolateral compartments. Data are presented as mean ± standard error mean (SEM) of at least 3 modules per case, from two independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ using either unpaired two-tailed test (for differences between two compartments in the same module) or one-way ANOVA followed by Tukey's multiple comparison test (for differences between treatments and different modules).

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In preparation for submission

CHAPTER VI

LOOKING INTO THE SAFETY OF CONDITIONALLY IMMORTALIZED CELLS FOR CELL-BASED THERAPEUTIC APPLICATION: A TALE OF RENAL REPLACEMENT THERAPY

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ABSTRACT

Despite five decades of improvements in dialysis treatment, end-stage renal disease still constitutes a global socioeconomic burden. Novel therapies that seek to integrate renal proximal tubule epithelial cells (PTEC) into dialysis filtration systems, termed bioartificial kidney (BAK), are being developed. Here, we investigated whether conditionally immortalized PTEC (ciPTEC) are safe for use in BAK systems, by focusing on their tumorigenic potential and chromosomal stability. We demonstrate that these cells, of which the function was enhanced through overexpression of the organic anion transporter 1 (OAT1), do not possess key properties of oncogenically transformed cells, including anchorage-independent growth, lack of contact inhibition and apoptosis-resistance. Moreover, time-lapse imaging of ciPTEC-OAT1, confined to a 3D extracellular matrix (ECM)-based environment, revealed that the cells were largely non-invasive, despite their chromosomal instability. The latter was observed in late-passage cells, favoring near-tetraploidy with complex chromosomal abnormalities. In addition, we determined the viral integration sites associated with the transgenes used for immortalization and cell function enhancement, and found the intronic regions of six distinct endogenous genes to be affected, among which early endosome antigen 1 (*EEA1*) and BCL2 Like 1 (*BCL2L1*) involved in endocytosis and apoptosis, respectively. Nevertheless, both gene products appeared to be functionally intact. Finally, after subcutaneous injection in athymic nude rats we show that ciPTEC-OAT1 lack tumorigenic and oncogenic effect *in vivo*, which is in agreement with the *in vitro* findings. Taken together, our study lays an important foundation towards BAK development by confirming the safety of the cell line intended for incorporation.

Keywords: bioartificial kidney; conditionally immortalized proximal tubule epithelial cells; SV40 Large T antigen; tumorigenicity; oncogenicity; chronic kidney disease; athymic nude rats; viral integration; endocytosis.

INTRODUCTION

The kidney plays a central role in maintaining homeostasis, excretion of xenobiotics and metabolic waste products, regulation of blood pressure, and several endocrine processes, such as vitamin D activation. It is therefore not surprising that failure of this organ can have severe consequences. End-stage renal disease (ESRD) represents the irreversible kidney failure and the final stage of chronic kidney disease (CKD) through a variety of causes. Ageing of the population, with frequently occurring diabetes and hypertension, is responsible for an increasing prevalence of ESRD [1-3].

Despite the large socioeconomic impact of ESRD [4], innovative novel therapies have thus far failed to reach the clinic. Over the last five decades, dialysis and transplantation have remained the backbone of renal replacement therapy [2]. Though these therapies have significantly improved the outlook of patients, limitations including a vast shortage of donor organs and complications related with immunosuppressive therapy could not be overcome [5,6]. Also dialysis therapy has

its drawbacks, related to an incomplete clearance of uremic solutes. This counts especially for the larger and protein-bound molecules, and the consequent accumulation of these molecules in the circulation has been associated with comorbidities, such as cardiovascular disease and cognitive impairment [7,8]. Recognition of these problems has spurred the development of novel approaches. Of promise are cell-based systems, also known as bioartificial kidney (BAK), that seek to replicate the kidney's function through the integration of renal cells, in particular proximal tubule cells [9].

One of the crucial issues to take into consideration when developing a BAK is the sufficient availability of suitable cells. Several studies focused on animal cells, like porcine primary proximal tubule epithelial cells (PTEC) [10-12], or cell lines such as the proximal tubule-like porcine cell line LLC-PK_1 (Lilly Laboratories cell porcine kidney 1) [13-15], and the dog-derived MDCK (Madin-Darby canine kidney) cell line [16,17]. The problem with using animal-derived cells in BAK is mostly related to safety concerns, the approval for clinical application and different behavior of animal cells. For instance, Ozgen *et al.* showed that LLC-PK_1 were able to form cell multilayers in hollow fiber membranes, with the risk of obstructing the device after two weeks of culture [14]. This suggests that animal-derived cells and cell lines, are far from being the ideal cell source for BAK. Human primary PTEC provide a suitable alternative. However, the limited life span of primary cells could abolish the concept of BAK, given that the number of cells needed for one device might be too high to be reached with expansion of primary cells. Additional risks associated with primary cells are functional changes that can occur during protracted culture, as well as the process of dedifferentiation and senescence [18,19], which would probably require the use of primary cells at specific passage numbers, reducing greatly the feasibility of BAK construction.

To alleviate the limited supply of primary cells, we employed urine-derived proximal tubule epithelial cells (PTEC) that were conditionally immortalized using the essential catalytic subunit of human telomerase (hTERT) and a temperature-sensitive mutant U19tsA58 of SV40 large T antigen (SV40T), creating conditionally immortalized PTEC (ciPTEC) [20]. These cells can be expanded at permissive temperature of 33 °C and differentiated into mature cells at non-permissive temperature of 37 °C [20-22]. While hTERT acts by stabilizing telomeres, thus preventing the occurrence of replicative senescence [23], SV40T involves the activation of E2F-mediated transcription through binding with Rb-E2F complex, as well as the inhibition of p53, which is caused by the conformational change of p53 and covering of the entire DNA-binding surface of p53 [24,25]. The cell line has been thoroughly characterized over the years [20,26-29], but the absence of the physiologically important organic anion transporter 1 (OAT1) protein led us to modify ciPTEC further by an overexpression of the transporter [30]. With this cell line we demonstrated the capacity of an efficient removal of uremic toxins when cells are cultured on hollow fiber membranes (HFM), thereby creating fully functional kidney tubules [31]. In addition to the demonstrated lack of ciPTEC allostimulatory potential *in vitro* [32], a successful upscaling of the biofunctionalized HFM with tight epithelial monolayers and cell function has been achieved, showing promising results and encouraging further efforts towards the BAK development [33].

Eyeing possible clinical applications of ciPTEC, a thorough safety evaluation is warranted to exclude any risks related to oncogenesis and tumorigenesis. Even though some studies suggest that SV40T mediated inhibition of p53 and Rb pathways or telomere length maintenance by hTERT are not sufficient to induce oncogenic transformation, various related parameters have to be examined [34-38]. Given that the oncogenes that were employed for conditional immortalization can lead to excessive cell proliferation, and that all transgenes involved were introduced by means of retroviral and lentiviral transductions, which are known to be able to drive oncogenesis [39,40], we here evaluate cell transforming properties and tumorigenic potential *in vitro* and *in vivo*, as well as genomic stability for the eventual disruption of endogenous genes by means of uncontrolled insertional mutagenesis [41].

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands) unless stated otherwise.

Cell culture

Both the parent cell line (ciPTEC) and its OAT1-overexpressing derivate (ciPTEC-OAT1) were maintained in culture as previously described by Nieskens *et al.* [30]. Briefly, cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1 DMEM/F-12) (Gibco, Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 5 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium, 35 ng/mL hydrocortisone, 10 ng/mL epidermal growth factor and 40 pg/mL tri-iodothyronine, creating complete culture medium, without addition of antibiotics. The cells were seeded at a density of 48,000 and 55,000 cells/cm^2, respectively. Seeded cells were kept at 33 °C for 1 day prior to 7 days of maturation at 37 °C. HeLa cells were propagated at 37 °C using high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco Life Technologies, Paisly, UK), supplemented with 10% (v/v) FCS and 1% (v/v) 5,000 U/mL penicillin/streptomycin, and seeded at a density of 55,000 cells/cm^2. All cell lines were passaged using Accutase® solution and incubated in a humidified atmosphere containing 5% (v/v) CO_2.

CiPTEC-OAT1 culture on hollow fiber membranes

To investigate cell proliferation in a 3D environment relevant to BAK, ciPTEC-OAT1 were cultured on microPES hollow fiber membranes (HFM; MicroPES type TF10 hollow fiber capillary membranes with wall thickness 100 µm, inner diameter 300 µm, max pore size 0.5 µm; Membrana GmbH, Wuppertal, Germany). Following L-DOPA (2 mg/ml) and collagen IV (25 µg/ml) double-coating, HFM were seeded with cells as described previously [31]. After 28 days culture at either permissive or non-permissive temperature HFM containing cells were fixed with 4% (w/v) paraformaldehyde (PFA; VWR International, Amsterdam, the Netherlands) dissolved

in PHEM buffer (120 mM PIPES, 50 mM HEPES, 4 mM $MgCl_2$, 20 mM EGTA) for 15 min and then washed in Hank's Balanced Salt Solution (HBSS; Gibco Life Technologies, Paisly, UK). Next, ProLong™ Gold antifade reagent containing DAPI (Life Technologies, Eugene, OR, USA) was used for nuclear staining, and to mount the fibers on the Willco glass bottom dishes (WillCo Wells B.V., Amsterdam, The Netherlands). Finally, cells were imaged using confocal microscope (Leica TCS SP8 X, Leica Microsystems CMS GmbH, Wetzlar, Germany) and analyzed by means of Leica Application Suite X software (Leica Microsystems CMS GmbH).

Contact inhibition

To investigate contact inhibition, ciPTEC-OAT1 and HeLa cells were cultured in 96-well plates (Costar 3599; Corning, NY, USA) at 33 °C and 37 °C for 4 weeks. The cells were, subsequently, fixed with 4% (w/v) PFA for 15 min and nuclei were stained with 1 μM Hoechst 33342 for 15 min. All aforementioned compounds were dissolved in phosphate-buffered saline (PBS; Lonza, Verviers, Belgium). A z-stack, spanning 100 μm with a 1 μm slice interval, was acquired on a Cell Voyager 7000 (CV7000) confocal microscope (Yokogawa Electric Corporation, Tokyo, Japan) with 10× magnification. At least 10 fields per condition were analyzed using ImageJ software 1.40 g. An ImageJ plugin was developed to quantify the surface area covered by cell multi-layers (Supplementary method 1), which signatures excessive proliferation and loss of contact inhibition. Values were expressed as percentage of total analyzed surface.

Cell cycle analysis

The effect of SV40T on the proliferation of ciPTEC-OAT1 was studied in a subconfluent and confluent state. To this end, cells were seeded at a low (10,000 cells/cm^2) and regular density and maintained in culture for various durations. Next, the harvested cells were fixed in ice-cold 70% (v/v) ethanol, washed twice with PBS, and incubated in a solution consisting of 40 μg/mL propidium iodide (PI), 0.1% (v/v) Triton X-100 and 100 μg/mL RNAse A for 30 min. DNA content of at least 10,000 cells per condition was measured on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and the cell cycle distribution was analyzed with FlowLogic 6.0 (Inivai Technologies, Melbourne, Australia).

Apoptosis evaluation

CiPTEC-OAT1 at permissive (33 °C) and non-permissive (37 °C) temperatures were exposed to nutlin-3a (Axon Medchem, Groningen, the Netherlands) to induce p53-mediated apoptosis [42,43]. Nutlin-3a was diluted from a 10 mM dimethyl sulfoxide (DMSO)-dissolved stock to desired concentrations. Following 24 h exposure, cell viability, as an indicator of apoptosis, was determined using PrestoBlue® cell viability reagent (Life Technologies, Paisly, UK). Briefly, cells were rinsed with HBSS and incubated with PrestoBlue® cell viability reagent for 1 h in the dark. Afterwards, the fluorescence was measured using a fluorescent microplate reader (Fluoroskan

Ascent FL, Thermo Fisher Scientific, Vantaa, Finland) at excitation wavelength of 530 nm and emission wavelength of 590 nm. The obtained values were corrected for background (absence of cells), normalized to untreated cells and presented as relative cell viability.

Soft agar assay

Colony-forming ability of cells in anchorage-independent conditions was assayed in a similar manner as previously described by Borowicz *et al.* [44]. Briefly, a 6-well plate (Costar 3506; Corning, NY, USA) was coated with 1.5 ml 0.5% (w/v) agarose that was diluted from an autoclaved 2% (w/v) agarose stock in cell culture medium. Upon solidification for 30 min at room temperature (RT), 10,000 ciPTEC-OAT1 or HeLa cells were mixed with the agarose stock solution to a final volume and concentration of 1.5 ml and 0.3% (w/v), respectively, and layered on top of the coating. After solidification, agarose layers containing cells were covered with 1 ml of complete culture medium and incubated for 4 weeks at either 33 °C or 37 °C. Colonies were manually counted in at least 15 fields per condition, using an Axiovert 25 bright-field microscope (Carl Zeiss, Oberkochen, Germany) at 10× magnification. Representative images of colonies were taken on CV7000 confocal microscope by phase-contrast microscopy equipped with a 20× objective.

Western blot analysis of SV40T

After seeding, ciPTEC-OAT1 were kept at 33 ˚C or matured for either 1 or 7 days at 37 ˚C. In addition to this, fully matured cells were also incubated for additional 4 h at 33 ˚C to simulate the effects of a transient temperature drop during hemodialysis. Following removal of culture medium and two washing steps in cold PBS, cell lysate was obtained through 5 min incubation on ice in 350 µl radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, Waltham, MA, USA), containing 1% (v/v) protease and phosphatase inhibitors (Halt Protease Inhibitor Cocktail, Halt Phosphatase Inhibitor Cocktail; Thermo Scientific, Waltham, MA, USA). Cell lysates were collected with a cell scraper (Costar 3010; Corning, NY, USA) and centrifuged at 14,000 × g for 15 min at 4 ˚C. The supernatant's protein content was, subsequently, determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) and samples were stored at -80 ˚C until further usage. Prior to Western blot analysis, cell lysates were denatured and reduced in 2% β-mercaptoethanol for 5 min at 95 ˚C using a T100 thermocycler (Bio-Rad, Veenendaal, the Netherlands). A total 15 µg of proteins per sample were separated by 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Veenendaal, the Netherlands) at 200 V and wet-transferred to a 0.2 µm pore-sized polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Veenendaal, the Netherlands) for 1 h at 100 V. The membrane was subsequently blocked for 1 h with 5% fat-free dry milk (Nutricia, Zoetermeer, the Netherlands) in PBS/0.1% Tween-20 (PBS-T; Millipore, Etten-Leur, the Netherlands). To allow protein quantification, the membrane was probed with mouse anti-SV40T (1:2,000; Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit anti-GAPDH (1:5,000) primary antibodies, followed by HRP-conjugated secondary antibodies for SV40T (1:5,000, rabbit anti-mouse; Dako, Carpinteria, CA, USA) and GAPDH (1:5,000, goat anti-rabbit; Dako, Carpinteria, CA, USA). Finally, chemiluminescence

was developed with Clarity Western ECL Substrate (Bio-Rad, Veenendaal, the Netherlands), images acquired using ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) and data analyzed by means of Image Lab software (version 5.2, Bio-Rad Laboratories). SV40T expression was quantified using Fiji software through densitometric analysis of imaged bands [45], using GAPDH for normalization. Data were presented as relative expression, using as a reference cells grown at permissive temperature.

Cytogenetic analysis

Metaphase spreads of ciPTEC-OAT1 were G-banded and analyzed for abnormalities (Cell Guidance Systems, Cambridge, UK). For sample preparation, log-phase cells (approximately at 70% confluence) were growth-arrested by treatment with 0.1 μg/ml colcemid (KaryoMAX, Gibco Life Technologies, Paisly, UK) at 37 °C for 15 h. Next, the harvested cells were treated with a hypotonic solution consisting of 75 mM KCl in HBSS. This was followed by fixation in 3:1 (v/v) methanol/acetic acid solution. To assess chromosomal stability, the experiment was performed twice over a period spanning 10 cell culture passages. Approximately 20 metaphase spreads were analyzed per experiment.

Single cell invasion assay

The invasion assay was based on the protocol described by Zaman *et al.* [46]. Matured ciPTEC-OAT1 were harvested, washed twice in HBSS and resuspended in serum-free medium (SFM). 125 μl of cell suspension, containing 40,000 cells in SFM, was thoroughly mixed with an equal volume of growth factor reduced Matrigel™ (Corning, NY, USA) and transferred to a 24-well plate (Costar 2524; Corning, NY, USA). Special care was taken to prevent the formation of air bubbles, which could hinder imaging and affect cell behaviour [46,47], hence the final gel thickness was approximately 200 μm. Following 1 h incubation at 37 °C, the solidified gel was gently covered with 250 μl of complete culture medium, serving as a chemoattractant. Time-lapse imaging was performed using CV7000 confocal microscope under environmentally controlled conditions (humidified, 5% (v/v) CO_2 and 37 °C), by taking pictures at 30 min intervals for 24 h. Each acquisition entailed a phase-contrast z-stack across the entire height of the gel, captured at 20× magnification with a slice thickness of 5 μm. Cell tracking analysis was performed using Fiji's TrackMate plugin to determine the speed of motion across acquisitions, as well as the average speed throughout the experiment [45,48]. Cells having a speed of > 6 μm/h were classified as invasive. We manually validated this threshold for optimum discrimination between invasive and non-invasive cells. Inclusion and exclusion criteria were applied as previously described [46]. All data were processed in MySQL 5.6.17 (Oracle, Redwood City, CA, USA).

Targeted locus amplification for viral integration sites

The ciPTEC-OAT1 cell line was stably transduced using three viral vectors [20,30], warranting an investigation into the occurrence of cell behavior-altering insertional mutagenesis. Determination of the exact location of the integrated transgenes was performed by Cergentis B.V. (Utrecht, the

Netherlands) using targeted locus-amplification (TLA) technology as described previously [49]. This technique enriches target genes and neighboring regions, enabling the detection of transgene integration sites and accurate mapping of single nucleotide variants and structural variants within the transgenes. Data was analyzed using Ensembl's genome browser in conjunction with the regulatory build [50,51]. In order to predict the functional consequences of the integration sites, the Ensembl Variant Effect Predictor (VEP) was utilized [52].

Endocytosis

To study endocytosis, bovine serum albumin (BSA) uptake and early endosome antigen (EEA1) were evaluated. First, ciPTEC-OAT1 and ciPTEC cells were seeded in black special optics 96-well plates (Corning, NY, USA) and exposed to 5 μg/ml BSA conjugated to Alexa Fluor 647 (Thermo Scientific, Waltham, MA, USA) for 1 h at 37 °C. Nuclei were simultaneously stained with 1 μM Hoechst 33342. A 20 μm z-stack was acquired at 0.5 μm intervals using the CV7000 confocal microscope at 60× magnification (excitation at 405 nm and 640 nm, acquisition using 445/40 nm and 676/29 nm band-pass (BP) filters, respectively). Afterwards, the cells were fixed with 4% (w/v) PFA and permeabilized using 0.1% (v/v) Triton X-100. Following 30 min blocking in 1% (w/v) BSA at RT, the EEA1 was probed with a rabbit anti-human EEA1 primary antibody (1:200; Cell Signaling, Danvers, MA, USA) and donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:500; Invitrogen, Carlsbad, CA, USA). For imaging, the CV7000 microscope with the same z-stack settings, excitation laser 488 nm and acquisition channel 525/50 nm BP filter was used to image EEA1 expression in cells. Finally, uptake of the fluorescently labelled BSA and EEA1 expression were both quantified using Columbus™ Image Data Storage and Analysis software 2.7.1 (PerkinElmer, Groningen, the Netherlands) and expressed as number of spots per cell, average spot size and spot intensity.

Tumorigenicity and oncogenicity evaluation in vivo

Male and female athymic nude rats (Hsd:RH-Foxn1^{rnu}; Envigo, Horst, Netherlands), maintained in the Central Laboratory Animal Research Facility (GDL, Utrecht, Netherlands), were used for *in vivo* tumorigenicity and oncogenicity studies. The rats were housed in individually ventilated cage units at RT under 12 h light/dark cycle. Food and water were provided *ad libitum*. Animal procedures were approved by the Ethics Committee of Animal Research of Utrecht University, Utrecht, The Netherlands (CCD approval number AVD108002017879). All animals were treated according to IVD and CCD guidelines and all efforts were made to minimize suffering. All animals were euthanized by pentobarbital (Faculty of Veterinary Medicine, Utrecht, the Netherlands) overdose via intraperitoneal injection, followed by cervical dislocation as soon as animals became unconscious.

The 5 weeks old rats, weighing between 94 and 172 g were randomly divided into four treatment groups keeping the ratio of male and female rats 1:1 in each group. The first test group received ciPTEC-OAT1 cells for tumorigenicity evaluation, the second group of animals received ciPTEC-OAT1 cell lysates, which were obtained by repetitive freeze/thaw cycles, to study oncogenic potential of ciPTEC-OAT1. For both tumorigenicity and oncogenicity groups,

ciPTEC-OAT1 were used at passage 57. The remaining two groups served as a positive control group that received HeLa cells and a negative control group that was given HBSS to monitor for spontaneous tumor growth. A total number of 10^7 cells or lysate derived from the equivalent amount of cells was injected subcutaneously in the flank of the animals in a total volume of 100 µl of HBSS, using 1 ml syringes with a 25 G needle (Terumo Europe N.V., Leuven, Belgium). The rats in the negative control group were injected with 100 µl of HBSS. Injections were performed in random order and in blinded fashion for the operator. After injection, all rats were observed for a maximum of 23 weeks, during which the animals were palpated every two days to detect nodule formation and tumor development at the site of injection and to monitor for clinical symptoms (weight, anxiousness, paralysis and diarrhea). Moreover, all animals were checked daily for their overall well-being. As soon as the tumors reached measurable size, the tumor length and width were measured using a calliper. Tumor volume was calculated using the formula: $1/2 \times \text{length (mm)} \times (\text{width (mm)})^2$. The rats were euthanized and necropsied when the humane endpoint (tumor mass of 4.2 cm in diameter or weight loss of more than 15% in two days) was reached, or when the tumors were of a sufficient size to consider them as progressively growing tumors. The site of injection, lungs, mesenteric lymph nodes, liver, spleen, kidneys and colon were harvested and examined by the Department of Pathology (Faculty of Veterinary Medicine, Utrecht, the Netherlands). Immediately after harvesting, tissue and tumor samples were fixed in formalin or frozen in liquid nitrogen until further use.

Histopathological analysis

First, formalin-fixed tissues and organs were examined macroscopically for the presence of abnormalities. Afterwards, formalin-fixed tissues were paraffin-embedded and slices of approximately 5 µm thickness were stained with hematoxylin and eosin and subjected to further microscopical histological analysis as described previously [53]. Representative images were taken using Olympus BX46 microscope equipped with DP25 camera (Leiderdorp, the Netherlands) with 2× magnification. Histologically confirmed tumors were further characterized by PCR.

DNA extraction and PCR analysis

Human origin of observed tumors was confirmed by detection of human specific *Alu* elements, the short interspersed elements (SINEs) present in primate genomes [54], using Real-Time PCR. After being recovered from liquid nitrogen, tumor tissues were cut into small pieces weighing between 20 and 25 mg and used for DNA extraction. Following tissue homogenization in PBS, genomic DNA was extracted using QIAamp® DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions and quantified using the NanoDrop® ND-1000 spectrophotometer. Next, Real-time PCR was performed using 25 ng of each DNA sample. The reaction was carried out using the iQ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) as indicated in manufacturer's protocol and by means of CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The reaction was performed by initial denaturation at 95 °C for 10 min, 40 amplification cycles at 95 °C for 15 sec, 62 °C for 5 sec and 72 °C for 15 sec, and followed by determination of melting curves by performing denaturation at 95 °C for 30 sec, followed by

complete annealing and a gradual increase in temperature starting from 60 °C and reaching 95 °C with a transition rate of 0.1 °C/sec, as described previously [55]. The data were analyzed using Bio-Rad CFX Manager™ Software version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) and expressed as mean Ct values. Specific sense and anti-sense primers for AluYb8 element [56] (forward: CGAGGCGGGTGGATCATGAGGT; reverse: TCTGTCGCCCAGGCCGGACT) were synthesized by Biolegio (Nijmegen, The Netherlands). To avoid non-specific rodent DNA amplification, low primer concentration of 150 nM was used. In each PCR reaction a negative control, given by rat genomic DNA, and a blank sample containing water instead of DNA were included. This was used also to determine the limit of the blank which represents the highest signal obtained in samples containing no human DNA [57]. Primer specificity and linearity of the PCR assay were determined by 5-fold dilutions of the known human DNA sample.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA), unless stated otherwise. Data are presented as the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate, unless stated otherwise. Significance was evaluated using the unpaired two-tailed Student's t-test or one-way ANOVA followed by Dunnett's multiple comparison test where appropriate. P-values < 0.05 were considered as significant. Where appropriate, significance is denoted as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

RESULTS

Conditionally immortalized PTEC proliferate and undergo apoptosis as a function of SV40T expression

First, the expression of SV40T was measured in ciPTEC-OAT1 under various conditions of cell culture. As expected and shown previously [20], the expression of SV40T was abundant at permissive temperature. However, after culturing cells at a non-permissive temperature as short as 1 day, the expression was reduced by approximately 90% and remained low for up to 7 days if cells were kept at 37 °C (Figure 6.1a). Though not the standard clinical practice in hemodialysis, various studies indicate that cooling the dialysate down to 35 °C can be beneficial, as it may enhance hemodynamic stability of the patient by preventing intradialytic hypotension [58,59]. To explore whether SV40T would remain downregulated during this eventual transient drop in temperature during dialysis, fully matured cells were re-exposed to 33 °C for a period of 4 h. Obtained results showed that there was no significant increase in the expression of SV40T compared to cells incubated at 37 °C for 7 days (Figure 6.1a), indicating that cells maintained their differentiated phenotype.

Moreover, to check the effect of SV40T expression on cell proliferation, cell cycle analysis was performed either on subconfluent or confluent ciPTEC-OAT1 cells forming an epithelial monolayer. The results shown in Figure 6.1b indicate that in a subconfluent state there was a

significantly higher proportion of cells in S-phase at the permissive temperature (40.5% ± 1.7%) compared to incubation at 37 °C for 1 day (10.5% ± 2.0%) or 7 days (17.0% ± 3.4%). These results correspond with the protein expression of SV40T, confirming that the expression of SV40T is directly related with cell proliferation. Under conditions of full confluency (Figure 6.1c) the same trend was observed with a higher proportion of proliferating cells at the permissive (20.4% ± 1.9%) compared to the non-permissive temperature (6.3% ± 6.9% after 1 day at 37 °C and 12.1% ± 1.5% after 7 days at 37 °C).

To study the effects of SV40T on p53 activity, one of its main targets [24], and to show that cells that are matured long enough at non-permissive temperature present regular p53 tumor suppressive functions such as apoptosis, cells were exposed to increasing concentrations of nutlin-3a to trigger p53-mediated apoptosis. Following 24 h of exposure, matured cells displayed higher sensitivity to nutlin-3a compared to cells cultured at permissive temperature expressing higher levels of SV40T. Indeed, cells at 33 °C were resistant to nutlin-3a induced cell death up to 25 μM, whereas cells at 37 °C showed reduced cell viability even after exposure to a low concentration of 10 μM. Cells cultured at 37 °C showed a higher cell death rate compared to cells at permissive temperature at all tested concentrations of nutlin-3a (Figure 6.1d-e).

CiPTEC-OAT1 obey the rule of contact-inhibition

Besides evaluating cell proliferation in relation to SV40T expression and conditional immortalization, ciPTEC-OAT1 were also investigated for their ability to undergo contact inhibition. For that purpose, ciPTEC-OAT1 were grown for 4 weeks at either permissive or non-permissive temperature, using HeLa cells as a positive control (Figure 6.2a). Results shown in Figure 6.2b-c indicate that ciPTEC-OAT1 growth did not extend beyond a confluent epithelial monolayer regardless of temperature, whereas HeLa cells presented multi-layered growth with more than 55% of growth surface covered with multiple cell layers when cells were cultured at 33 °C, and approximately 70% of multi-layered cell growth at 37 °C. In addition, when ciPTEC-OAT1 were cultured on L-DOPA and collagen IV coated HFM, no multi-layered growth was observed at either permissive or non-permissive temperatures (Figure 6.2d).

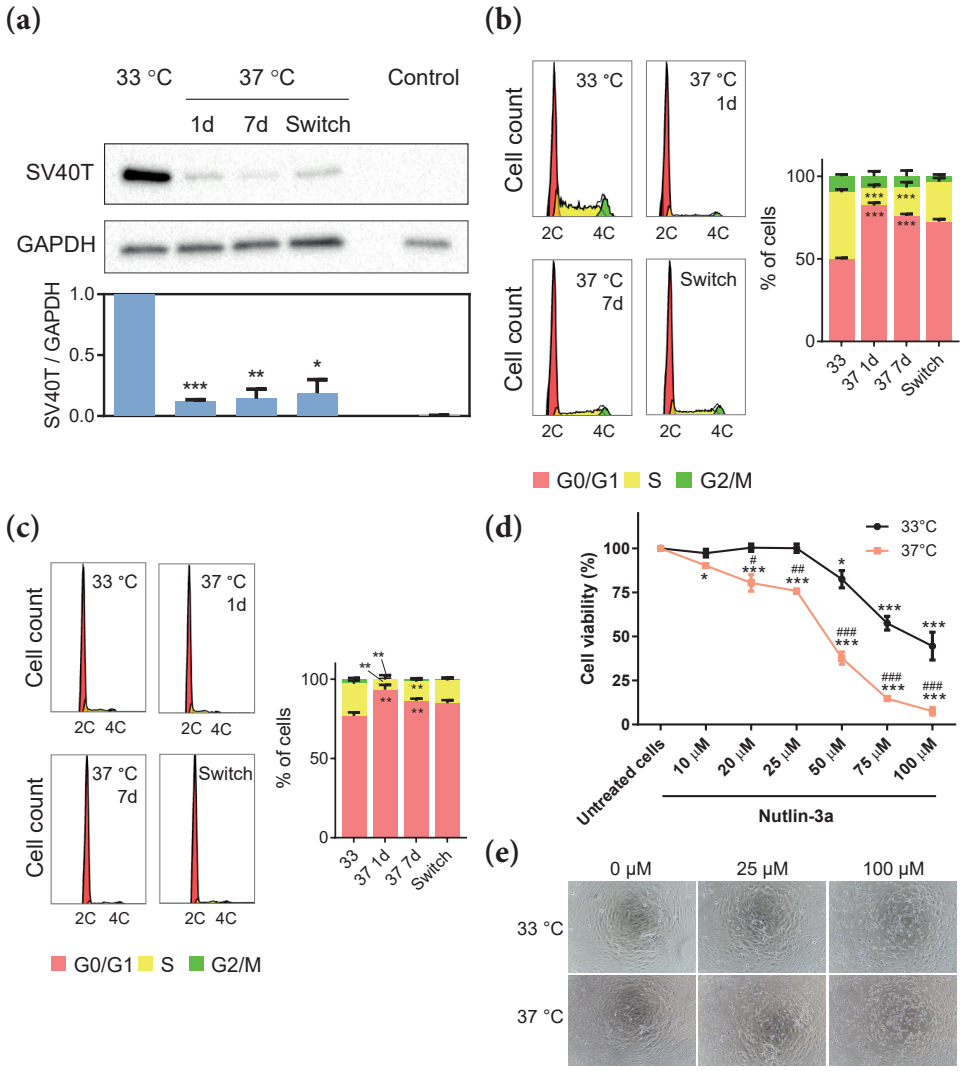


Figure 6.1. Temperature-dependent effect of SV40T expression on ciPTEC-OAT1 proliferation and apoptosis-sensitivity. (**a**) SV40T levels abruptly diminished when cells were transitioned from the permissive (33 °C) to the non-permissive (37 °C) temperature. Additionally, switching the temperature back to 33 °C for a period of 4 h did not result in a significant increase of SV40T. Proliferation of (**b**) subconfluent and (**c**) confluent ciPTEC-OAT1 followed a similar trend, with less proliferating cells at non-permissive temperature. In conditions of full confluency cells were underrepresented in the S and G2/M-phase as a result of contact inhibition. (**d**) Cell viability analysis of nutlin-3a treated cells shows that resistance to apoptosis is more pronounced at the permissive temperature. (**e**) Cell morphology at permissive and non-permissive temperatures upon exposure to nutlin-3a confirming higher resistance to cell death at permissive temperature (20× magnification). All values are expressed as the mean ± SEM of three independent experiments performed in triplicate.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired two-tailed Student's t-test and one-way ANOVA followed by Dunnett's multiple comparison test).

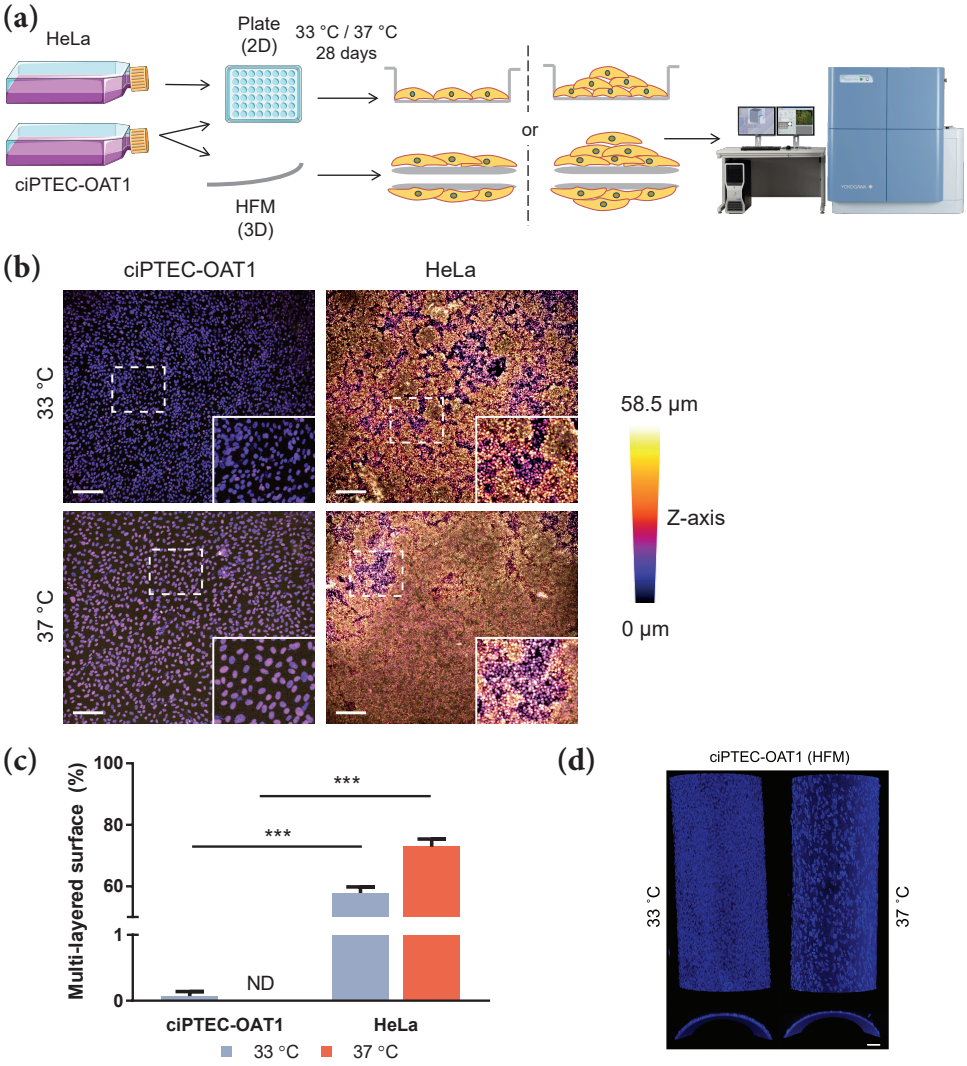


Figure 6.2. Contact inhibition in ciPTEC-OAT1. (**a**) Schematic diagram of focus formation assay. CiPTEC-OAT1 were cultured in 2D (96-well microplate) and 3D (hollow fiber membranes; HFM) for 28 days at 33 °C and 37 °C. HeLa cells were cultured in 2D in same conditions. Foci (multi-layered growth) formation was detected by nuclear staining and confocal imaging. (**b**) Depth-coded images of nuclei-stained ciPTEC-OAT1 and HeLa cells after 28 days of culture at both permissive and non-permissive temperature for ciPTEC-OAT1. Only HeLa cells displayed robust multi-layered proliferation. (**c**) Quantification of the surface area covered by multi-layered proliferation. ND = not detected. Scale bars denote 200 µm in the original image and 100 µm in the zoom-in. (**d**) Representative confocal images of nuclei stained ciPTEC-OAT1 cultured on double-coated HFM at 33 °C and 37 °C, x-y confocal planes on the upper part and y-z confocal planes on the bottom part. Images taken with 10× magnification. Scale bar: 50 µm. Values are expressed as the mean ± SEM of three independent experiments performed in triplicate. *** $p < 0.001$ (unpaired two-tailed Student's t-test).

CiPTEC-OAT1 require anchorage for proliferation

To assess whether ciPTEC-OAT1 possess the ability to proliferate in an anchorage-independent manner, a key hallmark of cancer cells [44,60], the soft agar assay was performed (Figure 6.3a). Single cells were encapsulated in semi-solid agarose medium thereby creating an environment that lacks the means for anchorage (i.e. cell-cell and cell-extracellular matrix interactions). Once again, cells were cultured at both 33 °C and 37 °C to evaluate whether SV40T could affect anchorage-independent growth. After 4 weeks of culture at the permissive temperature, sporadic colony formation was observed for ciPTEC-OAT1 (0.87 ± 0.21 colonies per field (CPF)) compared to control HeLa cells (12.70 ± 0.58 CPF). In a similar trend, colonies were absent in mature ciPTEC-OAT1 (0.07 ± 0.05 CPF) while abundantly present in HeLa (15.10 ± 0.95 CPF) (Figure 6.3b). From a qualitative perspective, the colonies that were incidentally seen in ciPTEC-OAT1 were of much smaller diameter than in the control HeLa cells (Figure 6.3c). This is well illustrated by the macroscopic images (Figure 6.3d), where only HeLa cells formed colonies at 37 °C that are large enough to be visible by eye. Taken together, these observations suggest that ciPTEC-OAT1 lose their proliferative capacity in absence of anchorage.

CiPTEC-OAT1 are predominantly non-invasive

To model invasive and metastatic capacity of ciPTEC-OAT1 *in vitro*, we performed a cell-tracking experiment in 3D environment consisting of growth factor reduced Matrigel™ basement membrane matrix. During the 24 h time-lapse, the majority of cells (87.5%) did not migrate and remained non-invasive (Figure 6.4a). However, a small population of cells was able to migrate through the extracellular matrix. Morphologically, these cells showed mesenchymal cell-like features and moved accordingly with a speed higher than 6 μm/h, whereas non-invading cells remained round-shaped with minimal movement within their own space at a speed below 6 μm/h (Figure 6.4a-c).

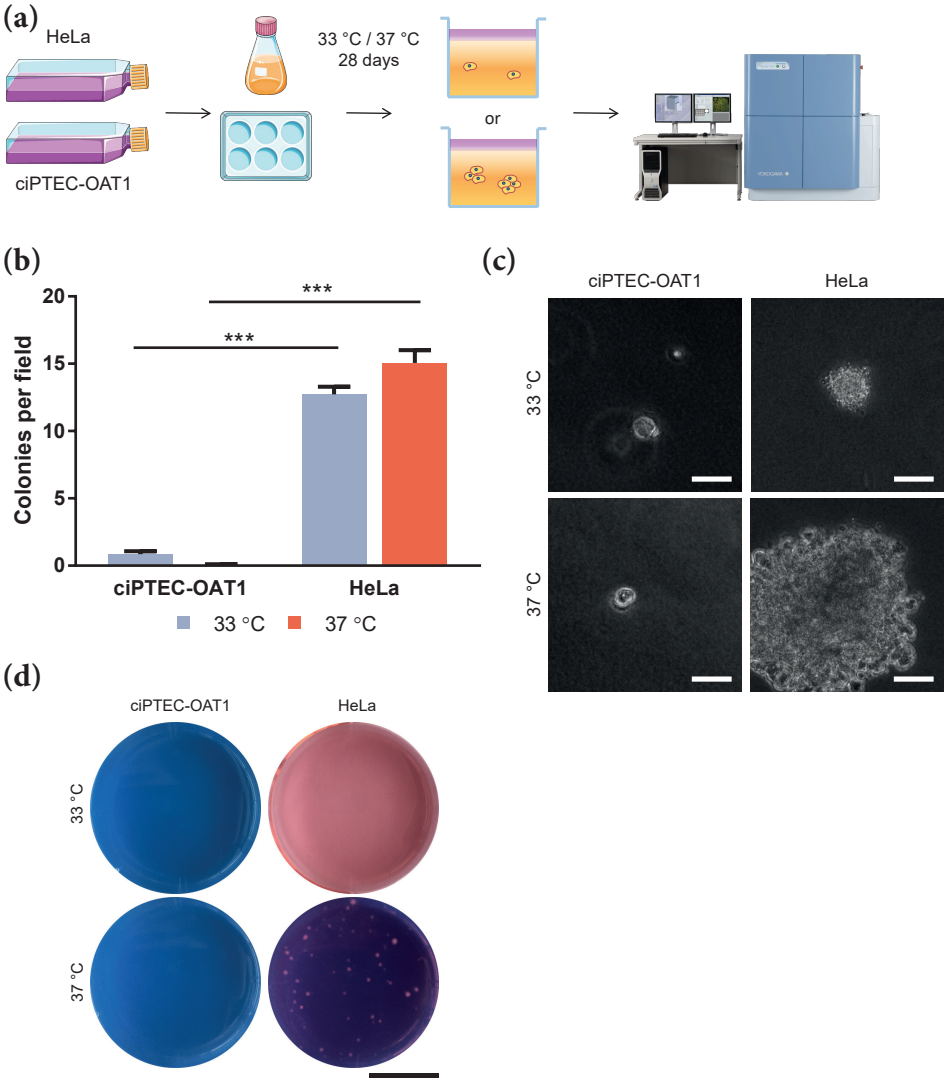


Figure 6.3. Anchorage-independent growth at permissive and non-permissive temperatures. (**a**) Schematic diagram of soft agar assay. Single ciPTEC-OAT1 and HeLa cells were seeded in agarose-containing medium (0.3% (w/v)) and incubated for 28 days at either 33 °C or 37 °C. Cell growth and colony formation was detected by confocal imaging. (**b**) The soft agar assay revealed that, regardless of temperature, ciPTEC-OAT1 virtually do not form colonies. (**c**) Besides the numerical differences, microscopic inspection showed that ciPTEC-OAT1 cell colonies were considerably smaller than HeLa cell colonies. Scale bars denote 50 μm. (**d**) From a macroscopic perspective, only HeLa cells at 37 °C grew abundantly enough to become visible as colonies. Scale bar denotes 1 cm. Values are expressed as the mean ± SEM of three independent experiments performed in triplicate. *** $p < 0.001$ (unpaired two-tailed Student's t-test).

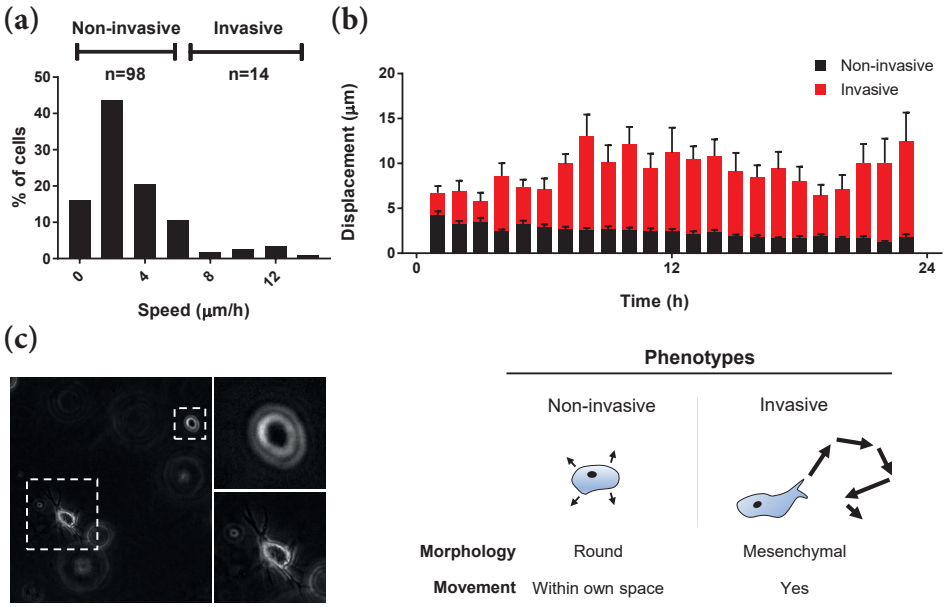


Figure 6.4. Invasive potential of ciPTEC-OAT1. (**a**) Histogram showing the average speed (µm/h) at which the cells moved. A bimodal distribution was observed, with most cells moving at a low speed. (**b**) Invasive and non-invasive cells showed a consistently different pattern of movement. (**c**) Morphological differences between the two phenotypes, acquired at 20× magnification. Invasive cells displayed mesenchymal cell-like movement, while non-invasive cells were round and static, wobbled only within their own space. Obtained values arise from 112 cells analyzed from two independent experiments.

VI

Transgene integration sites and genomic stability

Given the viral transductions that were involved in creating the ciPTEC-OAT1 cell line, it was important to understand how the stably integrated transgenes could affect endogenous genes and the genomic integrity of ciPTEC-OAT1. To fully comprehend the risk associated, TLA was utilized to amplify the transgenes and their surrounding regions, from which precise integration sites were mapped (Figure 6.5a). Transgenes integrations had occurred in the intronic regions of six endogenous genes. The gene of SV40T temperature sensitive mutant was shown to be integrated in the *GNA12* (chromosome 7) (Figure 6.5b; Figure 6.S1a,c) and *BCL2L1* (chromosome 20) (Figure 6.S1b,c) genes. The *hTERT* is stably integrated in the *CAMTA1* gene (chromosome 1) (Figure 6.S2), whereas the *SLC22A6* (encoding OAT1) transgene is integrated in the *WDR90* (chromosome 16), *KIAA1958* (chromosome 9) and *EEA1* (chromosome 12) genes (Figure 6.S3). Several predictions were made regarding the functional consequences of these insertion sites (Figure 6.5c). Except for *BCL2L1* and *EEA1*, all endogenous genes were located on the opposite strand compared to the inserted gene. Next, the evolutionary conservation of the affected sites, which is indicative of the importance of a sequence, was investigated. A moderate

degree of conservation was found for *BCL2L1* and *CAMTA1*, with phastCons 100-way scores of respectively 0.149 and 0.273. The other sites were classified as not conserved. In addition, all integration sites were analyzed with Ensembl's VEP. Solely the *BCL2L1* insertion site was found to be of relevance here, containing both a regulatory-active site as well as being part of an antisense sequence (Figure 6.5c). The remainder transcript types were classified as having a low probability of being functionally relevant, as they are either designated for degradation, such as nonsense-mediated decay (NMD), or the integrated sequence resides in a location where it would be spliced out.

For a final assessment of cell abnormalities, ciPTEC-OAT1 at the baseline passage of 52 were subjected to karyotype analysis. The cell population examined consisted of 68.2% near-tetraploid cells with the remainder being diploid. In-depth analysis of the diploid subpopulation (Figure 6.5d) shows the prevalence of an isochromosome abnormality concerning the p-arm of chromosome 7, i(7)(p10), which occurred in 2 out of 20 diploid metaphase spreads examined. To understand how these abnormalities can evolve over time, the cells were re-examined for their karyotype at passage 62. Here, a complete shift towards near-tetraploidy was observed, with the presence of various complex aberrances among the entire population examined.

Transgene integration does not affect endocytosis

Following the finding that the *EEA1* gene, whose protein product regulates endosomal trafficking in PTEC, is affected by a transgenic integration of the *SLC22A6*, a careful evaluation of its expression and function was performed. EEA1 is of particular importance because of its involvement in endosomal trafficking (Figure 6.6a), a crucial step in the reabsorption function of PTEC [61]. We show that endosomal clusters of EEA1, hereafter referred to as spots (Figure 6.6b-c), did not differ between ciPTEC-OAT1 and the control parent cell line that lacks the *EEA1*-affecting integration site, indicating that *EEA1* expression was maintained. To determine to what degree the function of EEA1 in endocytosis was affected in ciPTEC-OAT1, the uptake of fluorescently labelled BSA was evaluated. The obtained data indicate that BSA accumulated efficiently in ciPTEC-OAT1 (Figure 6.6b), similarly as in the control parent cell line. Spots indicating BSA uptake were analyzed and no meaningful differences were found in their properties compared to spots in control cells (Figure 6.6d), apart from the number of spots per cell, which appeared to be higher in ciPTEC-OAT1.

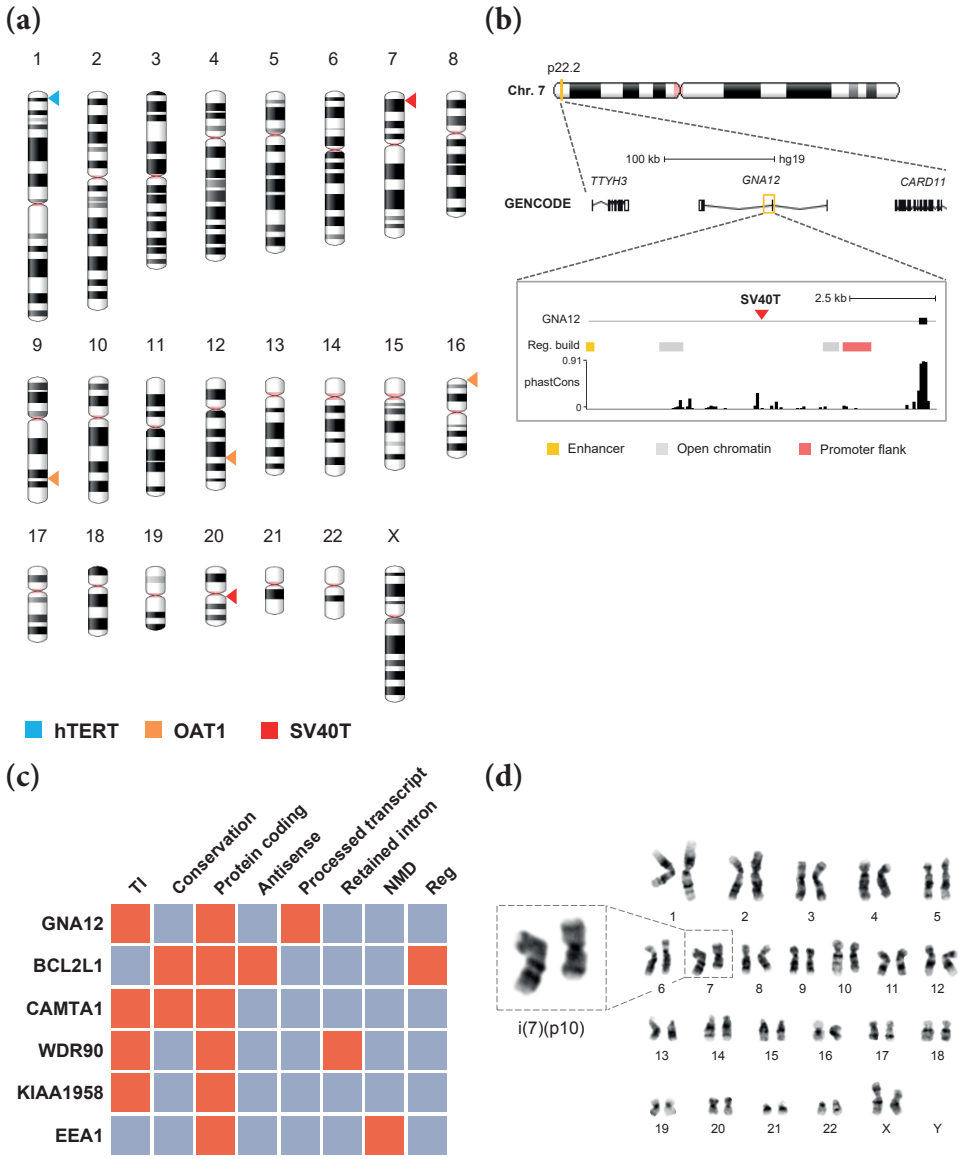
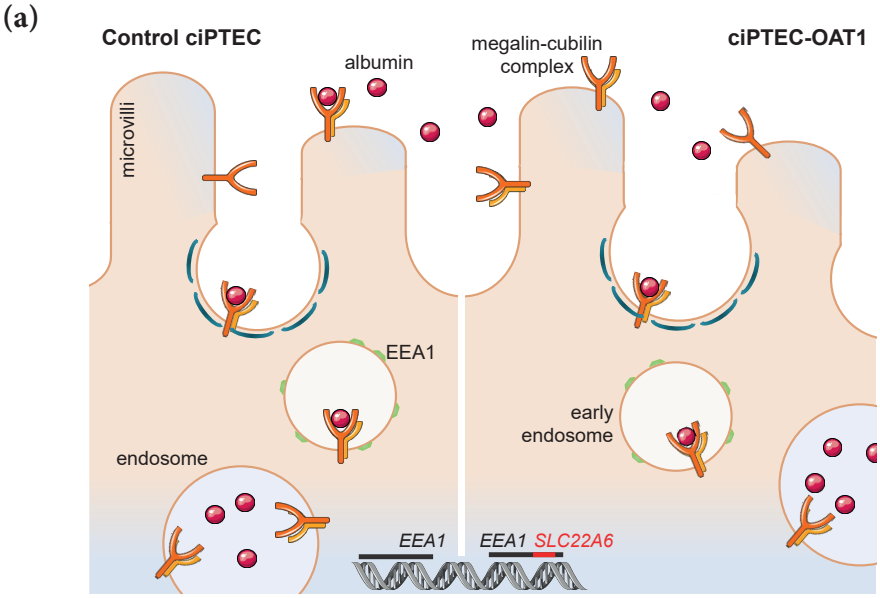
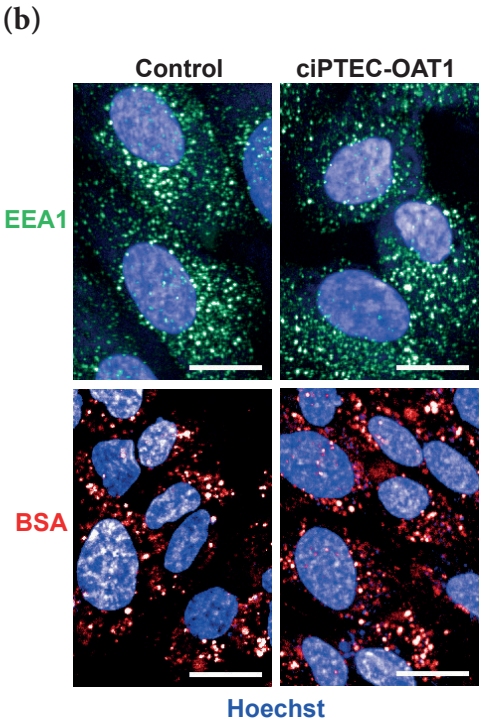
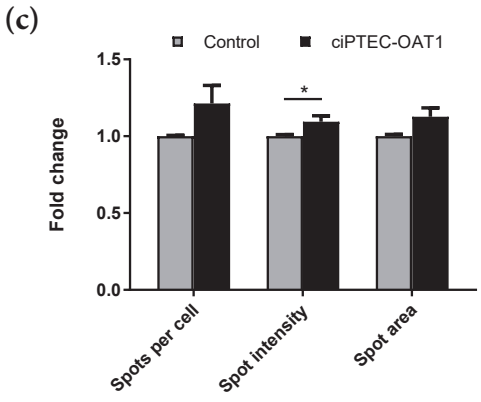


Figure 6.5. Viral integration sites and chromosomal stability. (**a**) Chromosomal distribution of the viral integration sites of the SV40T, hTERT and OAT1 transgenes. (**b**) The integration of SV40T gene into *GNA12*. See Figures 6.S1-6.S3 for schematic representation of remaining affected genes. Gene legend: untranslated region (empty box), exon (filled box), intron (line). (**c**) Functional consequence prediction of the viral integration sites. Presence and absence of a specific feature is shown in red and blue, respectively. Legend: TI = transcriptional interference, NMD = nonsense-mediated decay, reg = regulatory features. The phastCons P100 database was used to identify evolutionary conserved regions. (**d**) Cytogenetic analysis of ciPTEC-OAT1 at passage number 52. Representative female karyotype of a diploid cell showing an isochromosome for the short arm of chromosome 7.


(a)
Control ciPTEC
albumin
megalin-cubilin complex
ciPTEC-OAT1
microvilli
EEA1
endosome
early endosome
EEA1
EEA1 SLC22A6


(b)
Control
ciPTEC-OAT1
EEA1
BSA
Hoechst


(c)
Control
ciPTEC-OAT1
Fold change
1.5
1.0
0.5
0.0
*
Spots per cell
Spot intensity
Spot area

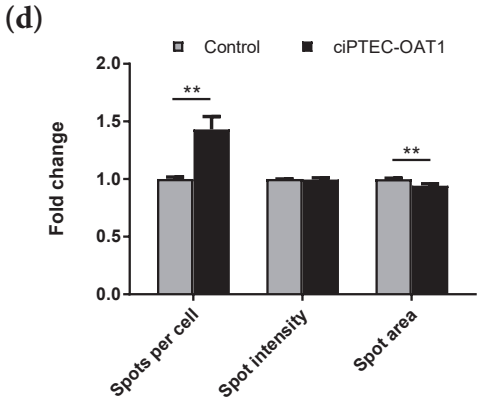
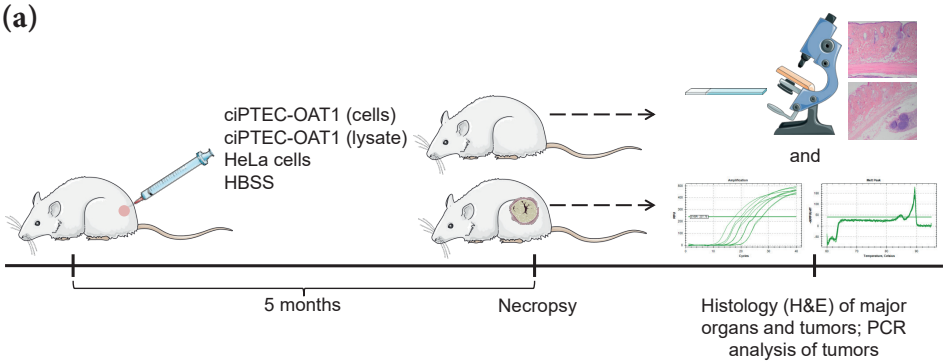

(d)
Control
ciPTEC-OAT1
Fold change
2.0
1.5
1.0
0.5
0.0
**
**
Spots per cell
Spot intensity
Spot area

Figure 6.6. Endocytotic capacity of ciPTEC-OAT1. (**a**) Schematic representation of receptor-mediated endocytosis of albumin by control ciPTEC and ciPTEC-OAT1, showing the integration of OAT1-encoding gene (*SLC22A6*) within *EEA1*. (**b**) Expression of the *EEA1* and the endocytotic uptake of Alexa Fluor 647 labelled bovine serum albumin (BSA). Scale bars denote 20 μm. Quantification of the spot properties showed similar (**c**) EEA1 expression and (**d**) BSA uptake compared to control (parent ciPTEC), but a higher number of BSA spots per cell in ciPTEC-OAT1. Values are normalized against control and expressed as the mean ± SEM of three independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ (unpaired two-tailed Student's t-test).

ciPTEC-OAT1 are not tumorigenic nor oncogenic *in vivo*

To evaluate whether ciPTEC-OAT1 exert tumorigenic and/or oncogenic effects *in vivo*, a study in nude athymic rats was performed according to the World Health Organization (WHO) guidelines [62]. Tumorigenicity was evaluated by subcutaneous injection of 10^7 living cells, and oncogenicity, representing the ability of subcellular elements, viral particles or genes to cause normal cells to transform into tumoral cells, was assessed by injection of cell lysates originating from the same amount of cells (Figure 6.7a). HeLa cells, used as positive control, produced palpable but not measurable nodules within the first week of injection in all rats. Over time, in 6 animals the nodules progressed into larger neoplastic masses that, by histological analysis, were confirmed to be anaplastic carcinomas in 5 out of 6 cases (Figure 6.7b, Figure 6.8b). In the remaining animal, the lack of histological confirmation of carcinoma was most likely attributable to a small-sized, non-measurable neoplastic mass at necropsy, which could have easily been missed in histological evaluation. PCR analysis confirmed the presence of human specific *Alu* elements in all identified carcinomas, and also in the rat without histological evidence of carcinoma (Figure 6.8e). Importantly, the subcutaneous injection of ciPTEC-OAT1 or its cell lysate did not lead to any nodule formation at the site of injection or in other distant places. Histopathological analysis showed no evident histological lesion in the skin, adnexa, subcutaneous tissue and lymph nodes at the site of injection, nor any neoplastic formations in other major organs including liver, lungs, colon, spleen, mesenteric lymph nodes and kidneys. This suggests that rats were not susceptible to spontaneous tumor formation and that ciPTEC-OAT1, in the given animal model, do not exert a tumorigenic or oncogenic potential.

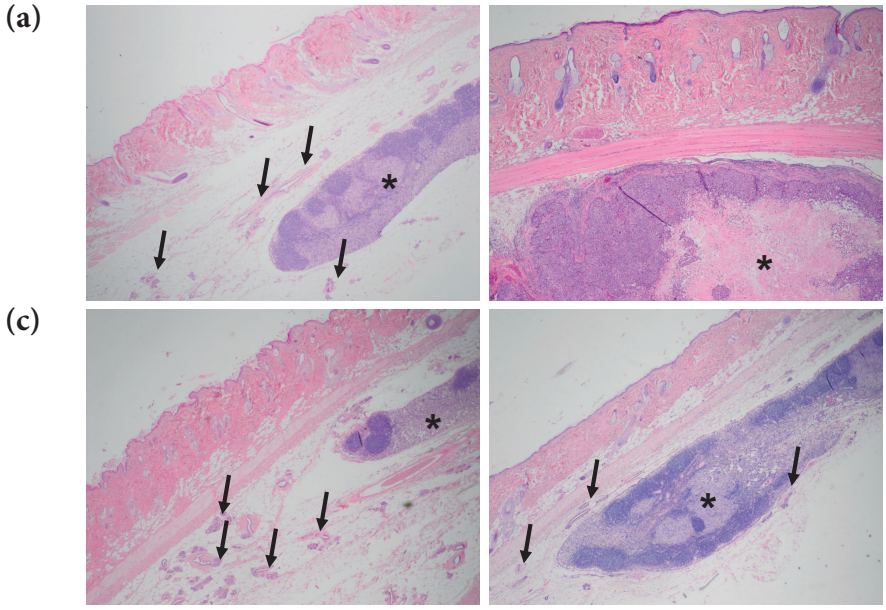
(a)



(b)

Test sample	Number of animals injected	Nodule at the injection site (during first week)	Neoplastic masses at the injection site (at necropsy)	Confirmed tumors at injection site (histology)	Confirmed *Alu* elements (PCR)	Tumors at other locations
HBSS (Negative control)	10	0	0	0	0	0
HeLa cells (Positive control)	10	10	6	5	6	0
ciPTEC-OAT1 cells	10	0	0	0	0	0
ciPTEC-OAT1 cell lysate	10	0	0	0	0	0

Figure 6.7. Tumorigenicity and oncogenicity of ciPTEC-OAT1. (**a**) Schematic representation of tumorigenicity and oncogenicity study *in vivo*. A total of 10^7 cells (ciPTEC-OAT1 or HeLa) resuspended in 100 µl of HBSS were injected subcutaneously in the flank of the rats. In an additional group cell lysate derived from 10^7 ciPTEC-OAT1 cells and resuspended in 100 µl of HBSS was injected per animal. In the negative control group rats received 100 µl of the vehicle (HBSS). Following the observational period of 5 months, histopathological and molecular (PCR) analyses were performed to confirm tumor formation and origin, respectively. (**b**) Summary of ciPTEC-OAT1 tumorigenicity and oncogenicity study results.



(e)

	Ct (mean ± SEM)	Tumor volume (mm^3) at necropsy
Tumor #1 (Rat ID n. 11)	17.2 ± 0.7	1,301
Tumor #2 (Rat ID n. 15)	19.3 ± 0.3	1,875
Tumor #3 (Rat ID n. 31)	20.2 ± 0.2	2,890
Tumor #4 (Rat ID n. 32)	26,8 ± 0,6	N.M.
Tumor #5 (Rat ID n. 33)	23.8 ± 0.7	2,145
Tumor #6 (Rat ID n. 35)	19.8 ± 1.2	309
Positive control (Human DNA)	18.3 ± 0.4	-
Negative control (Rat DNA)	>>35 (37.5 ± 0.4)	-
Blank (Water)	>>35 (37.2 ± 0.7)	-

Figure 6.8. Histopathological examination of injection sites and PCR analysis of tumor samples. Representative pictures of histopathological analysis of the injection sites, performed by eosin and hematoxylin staining. (**a**) Negative control group injection site showing normal skin with subcutaneous lymph node (*) and mammary tissue (arrows). (**b**) Positive control group injected with HeLa cells, showing the presence of anaplastic carcinoma with central area of necrosis (*) expanding in subcutaneous tissue at the site of injection. (**c**) Experimental tumorigenicity group injected with ciPTEC-OAT1 cells, presenting normal skin with subcutaneous lymph node (*) and mammary tissue (arrows). (**d**) Oncogenicity group injected with ciPTEC-OAT1 cell lysate, showing normal skin with subcutaneous lymph node (*) and mammary tissue (arrows). Pictures taken at magnification 2×. (**e**) PCR analysis of human-specific *Alu* elements confirming human origin of identified tumors and tumor volume (mm^3) at necropsy. Ct values of all tumor samples were similar to that obtained for positive control human genomic DNA. On the other hand, both the negative control (rat genomic DNA) and blank sample produced Ct values higher than 35. Limit of the blank [57], defined as the highest signal expected to be found when a blank sample containing no human DNA is tested, had a Ct value of 37.2 ± 0.71. N.M. (non-measurable).

DISCUSSION

In the present study, we mechanistically and functionally confirmed that ciPTEC-OAT1 behave in accordance with conditional immortalization. After culture at non-permissive temperature they lose proliferative capacity and show contact inhibition. We found no indication for an important effect of transgene genomic integrations on endogenous gene expression or function. Although chromosomal aberrations could be demonstrated after multiple cell culture passages, there were no signs of *in vivo* tumorigenicity nor oncogenicity.

The presence of residual SV40T, even after 7 days of culture at the non-permissive temperature, is in line with the known thermolabile characteristics of the used SV40T mutant, as it will only be completely inactivated above 39.5 °C [22]. Furthermore, considering BAK application and in case of an eventual temperature drop during hemodialysis treatment, we observed a slight, but not significant, rebound in SV40T expression after re-exposing fully matured cells for 4 h at 33 °C. We further experimentally confirmed that this has no apparent effect on cell proliferation. Moreover, we showed that cell proliferation is dependent on the SV40T expression as cells maintained at permissive temperature were highly proliferative compared to cells incubated at non-permissive temperature. This is in line with the mechanism of action of SV40T, which is known to interfere with Rb and p53 pathways thus driving cell proliferation [24]. Finally, cell ability to undergo p53-mediated apoptosis was also confirmed using nutlin-3a, a compound that selectively induces p53 by inhibiting its degradation via Mdm2 [42], at both permissive and non-permissive temperatures, suggesting normal p53 activity and apoptosis regulation at non-permissive temperature.

We examined the presence of contact inhibition in ciPTEC-OAT1 for two reasons. Firstly, non-transformed epithelial cells are expected to be responsive to growth-regulatory signals and undergo contact inhibition, meaning cell growth arrest when the surface of culture plastic is completely covered and cells are in contact with each other. Failure to display this would raise safety concerns as it would reflect cancerous cell behavior [60]. Secondly, eyeing the potential use of ciPTEC-OAT1 in a BAK device [31], overgrowth of the cell monolayer could result in clogging of the tubules of the BAK device or cause alteration of the epithelial monolayer barrier function. Our results showed that ciPTEC-OAT1 do undergo contact inhibition and do not grow beyond the expected monolayer, even when they are cultured for long periods of time at permissive temperature. Moreover, when cultured on double-coated HFM for future BAK application, ciPTEC-OAT1 did not form multiple epithelial layers at neither of the two temperatures evaluated. The observed absence of multi-layered growth of ciPTEC-OAT1 could also be explained by the presence of a crowding-induced live cell extrusion mechanism that helps maintaining homeostatic cell numbers in the epithelium [63].

Employing a soft-agar assay, known to correlate closely to *in vivo* tumor-forming ability of cells [64], we demonstrated that ciPTEC-OAT1 do not proliferate in an anchorage-independent manner, which indicates a lack of cell transformation and tumorigenic potential. Even though very small colonies were observed sporadically, these were not progressively growing colonies such as those formed by HeLa cells and their diameter was not larger than 35 μm. This clearly suggests that such identified colonies might have been small cell clumps, considering that single ciPTEC

cell diameter is approximately 11 μm [26]. Besides, regular studies assessing colony formation in soft agar are usually performed from a macroscopic perspective [44] with clearly visible colonies, and not with sensitive microscopic approach as used in the present study. From our results it is clear that HeLa cells grow in absence of anchorage as described for cancer cell types [60], while ciPTEC-OAT1 do not exhibit this feature.

Many assays have been described to study metastatic behavior of malignant cells *in vitro* [65]. A requirement for metastasis is that cells are capable of invasion, i.e. migration through an extracellular matrix (ECM) barrier. Due to steric hindrance imposed by the ECM, movement is primarily limited to the cell's ability to proteolytically degrade its surroundings, though amoeboid motility has also been reported to occur, depending on the (micro-)environment [46,66]. In our experiment, single ciPTEC-OAT1 cells were confined to a 3D environment that consisted of growth factor reduced Matrigel™, using FCS-containing complete cell culture medium as a chemoattractant. The majority of cells lacked signs of invasive behavior, though in a small subset of cells we observed mesenchymal cell-like movement through the gel during the 24 h incubation. In addition to being a property of malignant cells [60], studies have shown that this type of movement also plays an important role in normal physiological processes, notably tissue repair [67]. Furthermore, it should be emphasized that metastasis is a complex multi-step process, involving detachment from the cell bulk, intravasation into the systemic circulation, survival in a relatively harsh environment (e.g. lacking anchorage and presence of immune surveillance) and finally, extravasation into a distant tissue or organ [66]. No single *in vitro* assay fully recapitulates the complete chain of these events [65]. However, the physiologic relevance of this finding, regarding cell's capacity of migration and invasion in ECM-based environment, could be determined in the context of the *in vivo* study evaluating tumorigenic and metastatic potential of cells, as discussed later on.

Understanding the functional consequences of the viral integration of the SV40T gene, *hTERT* and *SLC22A6* transgenes in ciPTEC-OAT1 is far from trivial. In contrast to protein-coding sequences, the function of non-coding DNA remains largely unknown and the annotation of regulatory elements is often based on predictive models. Of such, Ensembl's Regulatory Build is a good example, taking into account epigenetic markers, transcription-factor binding sites and DNase I hypersensitive sites (DHS), amongst other features, to define regulatory regions [51]. The limitations become clear when considering the integration site of *BCL2L1*, a gene involved in both pro- and anti-apoptotic signaling through its two protein isoforms, Bcl-xS and Bcl-xL [68-70]. The Regulatory Build categorizes this area as a promoter-flanking region, despite its location being approximately 27 kb downstream from the actual promoter. To complicate things further, the activity of regulatory elements tends to be cell-type specific, which makes the estimation of impact through an *in silico* approach particularly challenging. Our results demonstrating that ciPTEC-OAT1 remain subjected to the intrinsic apoptosis pathway, of which Bcl2l1 is a key regulator [71], suggest that the viral insertion did not reduce the cell's capacity to undergo apoptosis. Another interesting observation is that four out of six transgenes are integrated in the opposite DNA strand. A difference in orientation can cause transcription machineries to converge and collide, a process termed transcriptional interference (TI). This generally manifests itself in

decreased transcript levels [72,73]. However, this type of integration also has a protective effect as it allows the transgene to be spliced out, resulting in an intact messenger RNA (mRNA) of the endogenous gene [72]. On the other hand, the transgenes of which the orientation matches that of the endogenous gene, which is the case for *BCL2L1* and *EEA1*, could potentially have a different effect. Namely, it has been described that transcription of the endogenous genes can be prematurely halted due to the presence of a termination signal in the long-terminal repeats (LTRs) of the viral vectors, leading to a truncated transcript of the endogenous gene [41]. However, in estimating the impact of the integrations sites, it should be noted that one healthy allele remains for each affected gene, possibly limiting the impact of integration. In support of this notion, we demonstrated an intact endocytotic capacity in ciPTEC-OAT1 proving the unaltered expression and function of *EEA1*. Overall, based on our results and prediction tools, we deem it very unlikely that the function of *EEA1* and *BCL2L1* is negatively affected in ciPTEC-OAT1.

We examined the chromosomal stability of ciPTEC-OAT1 by karyotyping through passages 52 and 62, and showed the presence of a growing subpopulation of near-tetraploid cells with various complex chromosomal aberrations. It has previously been shown that through its interaction with Bub-1, a spindle assembly checkpoint protein, SV40T can breach genomic integrity and induce tetraploidy [74]. Although this interaction appears to be unnecessary for immortalization, it has been demonstrated to trigger oncogenic transformation [75]. While the latter seemed absent as ciPTEC-OAT1 did not display key features of transformed cells, there is also a contradiction with other studies, including those underlying the creation of the ciPTEC-OAT1 line, where chromosomal abnormalities could be avoided [22]. In light of these discrepancies, our research thus supports the notion that generalizations cannot be made. SV40T as a tool for immortalization requires additional scrutiny, warranting a case-by-case evaluation of its impact on the chromosomal stability. This is especially important from a clinical perspective, as chromosomal instability could affect safety characteristics of a cell line. The creation of a SV40T mutant (U19dl89-97tsA58) that lacks the interaction site with Bub-1 is a promising development that seeks to avoid these shortcomings [76].

WHO guidelines regarding cell-based therapies suggest that all cell types intended for therapeutic purposes should be genetically stable as otherwise they would impose a significant risk regarding cell function and tumorigenic potential [62]. With regard to cell function, it should be noted that ciPTEC-OAT1 have previously undergone a rigorous functional assessment, showing that, at least from the functional perspective, cells remain stable over a wide range of passages [30]. Despite chromosomal aberrations, these cells were shown to be able to differentiate in mature cell monolayers performing a wide range of PTEC-related functions including epithelial barrier formation, protein uptake, vitamin D activation, as well as transport of uremic metabolites [30,31,77].

Other concerns described in the WHO guidelines are tumorigenicity, defined as the capacity of a cell population to form a tumor at the site of inoculation in an appropriate animal model, and oncogenicity, which represents the capacity of an acellular agent and intracellular components to cause normal cells of a host animal to produce tumors [62]. We addressed these issues for ciPTEC-OAT1 in accordance to the WHO regulations that indicated the total number of animals

per group to be inoculated, number of cells, administration route, passage number of inoculated cells, observation period, as well as the inclusion of proper controls. In case of oncogenicity, cell lysates were prepared by repeated freeze/thaw cycles to avoid disruption of potentially present viral particles while allowing maximum virus release and ensuring that all cells were completely lysed. In the present study, the negative control group (vehicle control) showed no tumor formations during the 5 months follow-up, arguing for the absence of spontaneous tumor formations. The guidelines further suggest that 90% of the animals within the positive control group should develop progressively growing tumors at the site of injection. Although within the first week after HeLa cells injections nodules appeared in all animals in the positive control group, we eventually observed that 50% of animals developed histologically and 60% PCR confirmed HeLa derived tumors. This indicates susceptibility of the animal model to grow tumor xenografts, even though not in complete accordance with WHO recommendations. Moreover, according to the guidelines, at least 20% of the animals within the test group should develop tumors in order to consider a particular cell type to be tumorigenic or oncogenic. Given that none of the animals developed tumors in the two test groups, we carefully conclude that in the athymic nude rat model, ciPTEC-OAT1 did not possess tumorigenic nor oncogenic potential. This is also in accordance with our *in vitro* results, suggesting the absence of tumorigenic phenotype of ciPTEC-OAT1.

Finally, considering that possible clinical use of these cells would only be in a context of an extracorporeal medical device and not direct transplantation, the altered karyotype and rare events of invasion observed *in vitro*, providing proper cell function, should not pose an extreme safety threat.

CONCLUSIONS

In summary, by showing that ciPTEC-OAT1 do not portray fundamental characteristics of oncogenically transformed cells, do not present negative consequences of viral transductions and genomic transgene integrations, such as insertional mutagenesis, nor possess tumorigenic capacity *in vivo*, the present study lays an important foundation towards validating the safety of a conditionally immortalized cell line for clinical application as cell-based therapy.

ACKNOWLEDGMENTS

This research was supported by the Marie Curie ITN project BIOART (grant no. 316690, EU-FP7-PEOPLE-ITN-2012). Milos Mihajlovic and Koen Westphal would like to thank Monika Verdouw for her help with animal handling during subcutaneous injections.

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51, 53-59.

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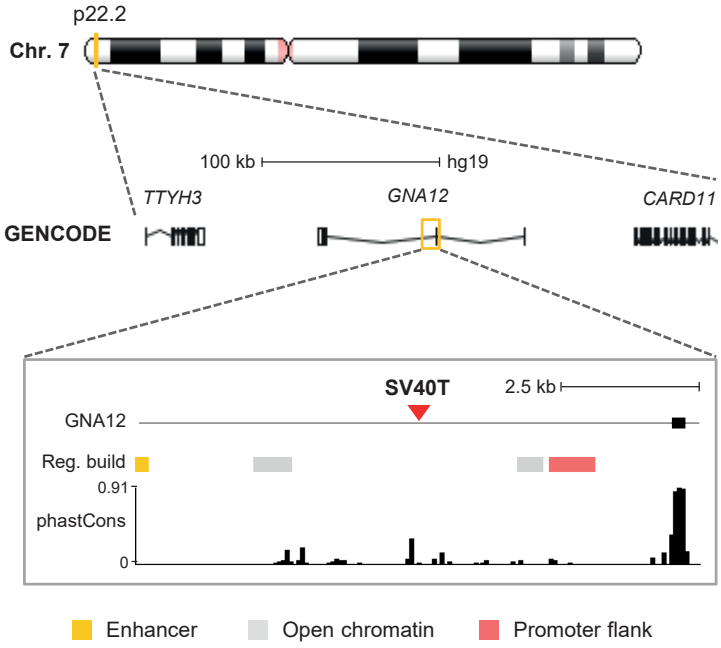
SUPPLEMENTARY INFORMATION

Supplementary method 1: ImageJ plugin for the quantification of surface area containing multi-layered cell growth.

```
// Step 1: Specify the name of the ImageJ window here
var windowName = "window";
selectWindow(windowName);
// Step 2: Convert to 8-bit
run("8-bit");
// Move up in the z-stack till single-layered cells are not visible
// Specify the slice (value depends on your experimental set-up)
setSlice(30);
selectWindow(windowName);
// Step 3: Enhance contrast
run("Brightness/Contrast...");
for (i = 0; i < 4; i++) {
        run("Enhance Contrast", "saturated=0.35");
}
run("Apply LUT", "stack");
selectWindow(windowName);
// Step 4: Blur the image to account for empty space between cells
// Remember, we're imaging nuclei, so inevitably there will be empty space (even in a fully confluent plate)
run("Gaussian Blur...", "sigma=10 slice");
selectWindow(windowName);
// Step 5: Threshold the image to get pixels with cells (white) and pixels without (black).
// The following code regarding thresholding is courtesy
// of Michael Schmid (Institut für Angewandte Physik, Technische Universität Wien)
percentage = 75;
nBins = 256;
resetMinAndMax();
getHistogram(values, counts, nBins);
nPixels = 0;
for (i = 0; i < counts.length; i++) {
 nPixels += counts[i];
}
nBelowThreshold = nPixels * percentage / 100;
sum = 0;
for (i = 0; i < counts.length; i++) {
 sum = sum + counts[i];
 if (sum >= nBelowThreshold) {
```

```
    setThreshold(values[0], values[i]);
    i = 99999999; // Break out of loop
  }
}
setOption("BlackBackground", false);
run("Convert to Mask", "method=Default background=Light only");
selectWindow(windowName);
getHistogram(values, counts, 256);
var results_c = nResults;
// Step 6: Output the results to the ImageJ console
setResult("Surface area containing multi-layered cell growth", results_c, counts[255]);
setResult("Total surface area", results_c, counts[0] + counts[255]);
updateResults();
```

(a)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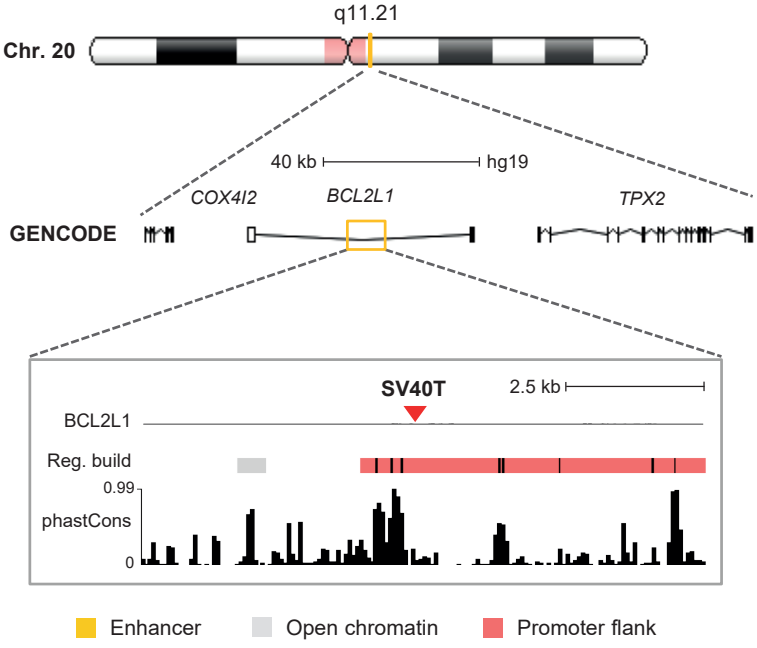
Chr. 7
100 kb
hg19
TTYH3
GNA12
CARD11
GENCODE
SV40T
2.5 kb
GNA12
Reg. build
0.91
phastCons
0
Enhancer
Open chromatin
Promoter flank

(b)


q11.21
Chr. 20
40 kb
hg19
COX4I2
BCL2L1
TPX2
GENCODE
SV40T
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BCL2L1
Reg. build
0.99
phastCons
0
Enhancer
Open chromatin
Promoter flank

(c)

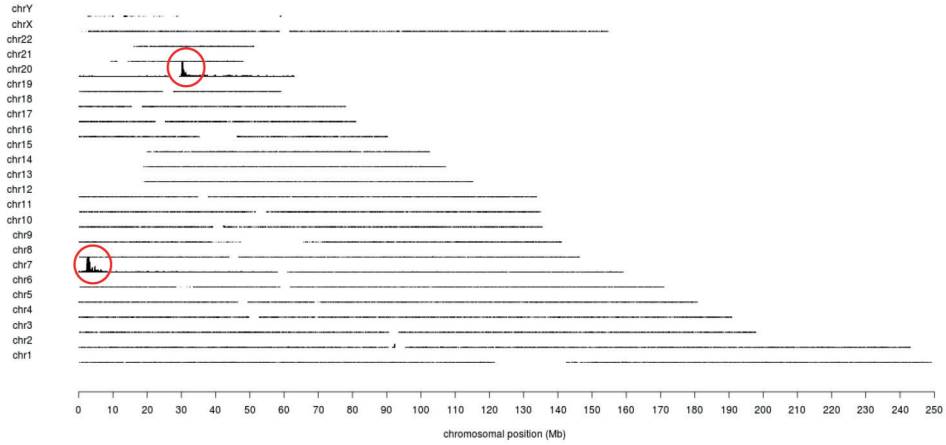


Figure 6.S1. Viral integration sites of theSV40T gene. The integration sites of the gene encoding SV40T within (**a**) *GNA12* and (**b**) *BCL2L1*.The phastCons P100 database was used to identify evolutionary conserved regions. Gene legend: untranslated region (empty box), exon (filled box), intron (line). (**c**) TLA sequence coverage across the human genome with different chromosomes indicated on the y-axis, and the chromosomal position on the x-axis. The position of the integration sites of the SV40T gene are encircled in red.

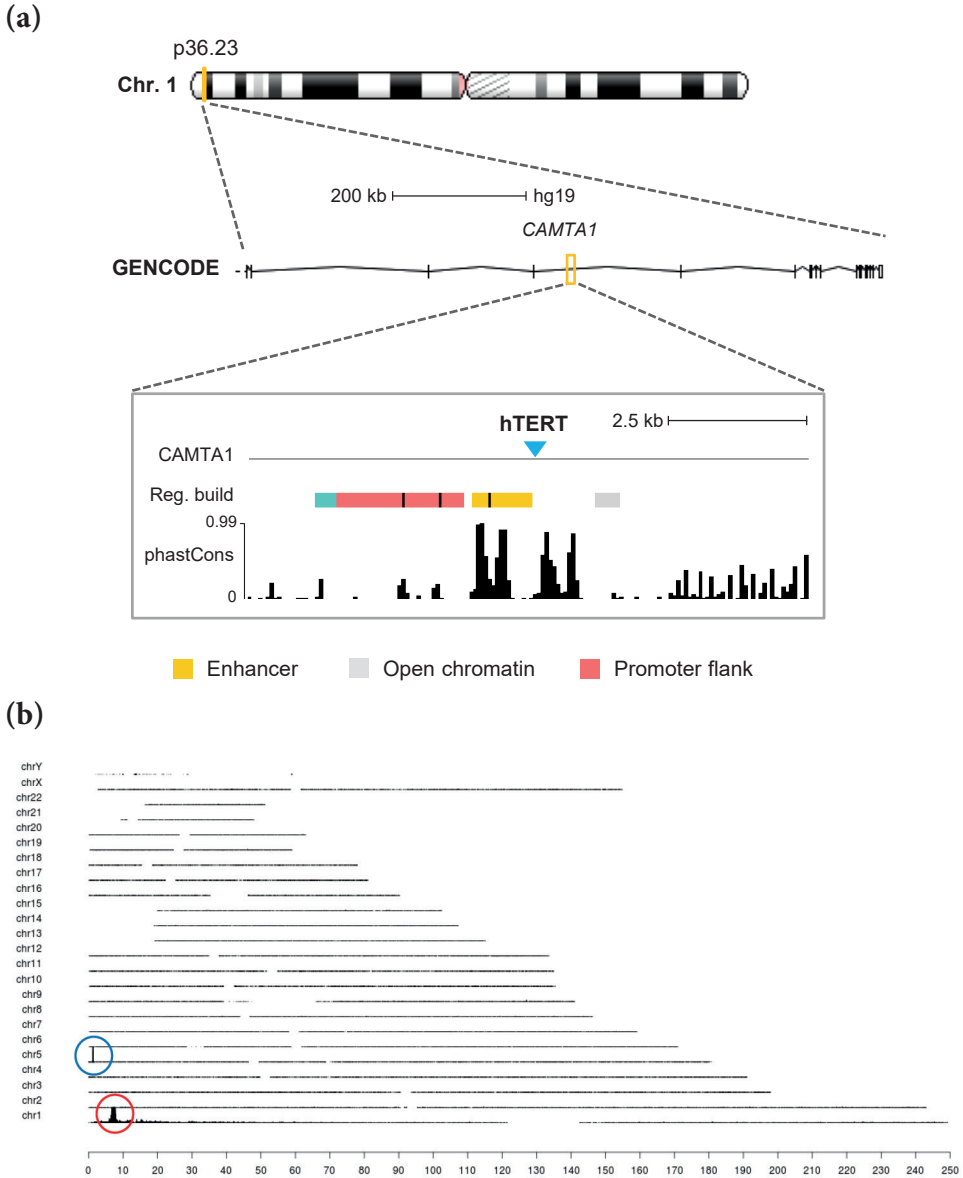
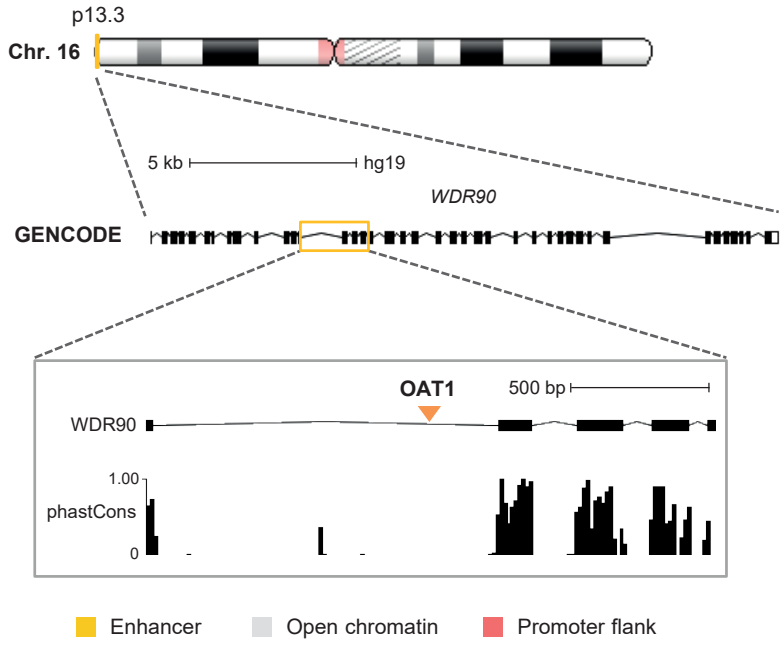


Figure 6.S2. Viral integration sites of hTERT. The integration sites of the gene encoding hTERT within (**a**) *CAMTA1*.The phastCons P100 database was used to identify evolutionary conserved regions. Gene legend: untranslated region (empty box), exon (filled box), intron (line). (**b**) TLA sequence coverage across the human genome with different chromosomes indicated on the y-axis, and the chromosomal position on the x-axis. The position of the integration site of *hTERT* is encircled in red. Encircled in blue is the position of hTERT gene locus.

(a)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500 bp
WDR90
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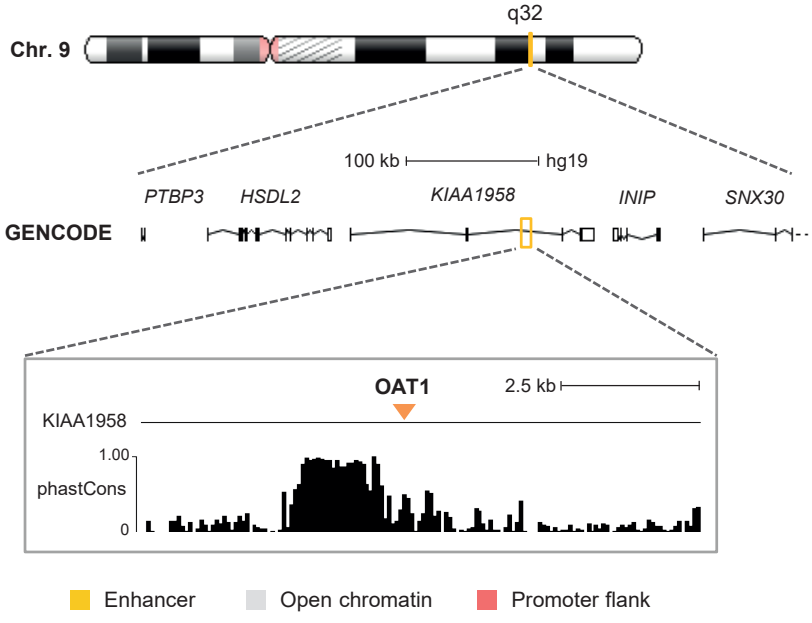
phastCons
0
Enhancer
Open chromatin
Promoter flank

(b)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HSDL2
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phastCons
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Enhancer
Open chromatin
Promoter flank

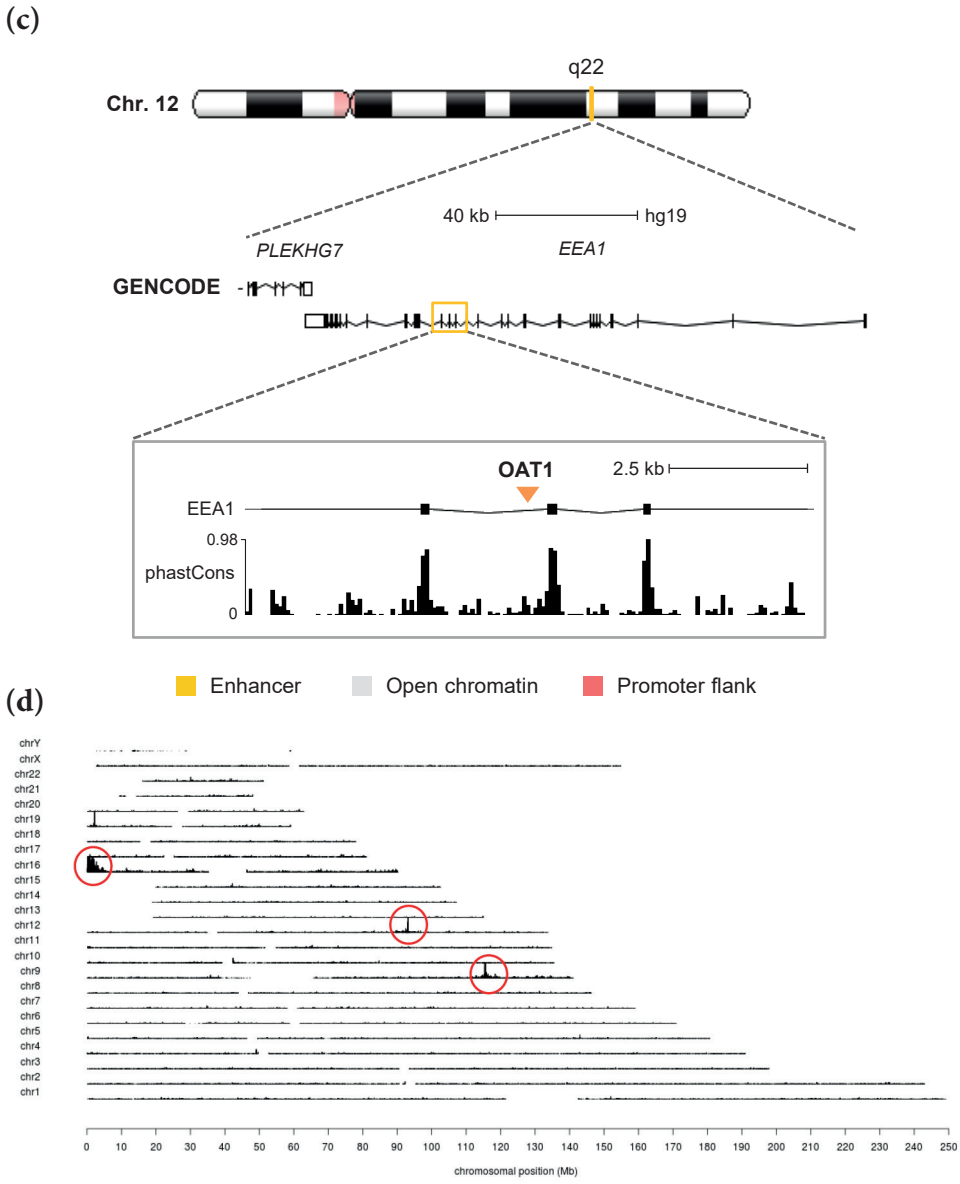
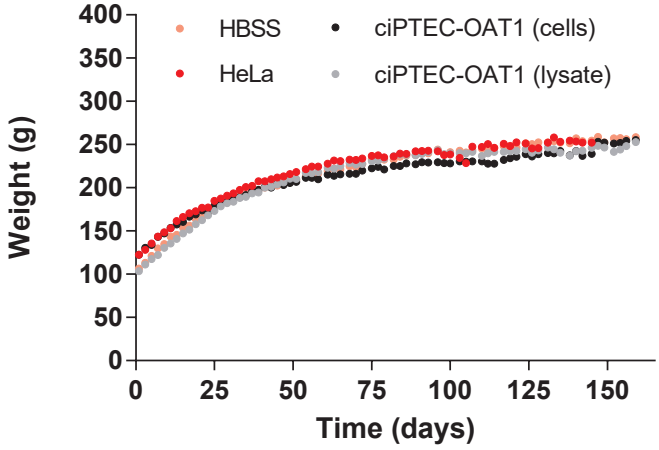


Figure 6.S3. Viral integration sites of SLC22A6 (encoding OAT1). The integration sites of the gene encoding OAT1 within (**a**) *WDR90*, (**b**) *KIAA1958* and (**c**) *EEA1*.The phastCons P100 database was used to identify evolutionary conserved regions. Gene legend: untranslated region (empty box), exon (filled box), intron (line). (**d**) TLA sequence coverage across the human genome with different chromosomes indicated on the y-axis, and the chromosomal position on the x-axis. The position of the integration sites of the OAT1-encoding gene are encircled in red.

(a)



(b)

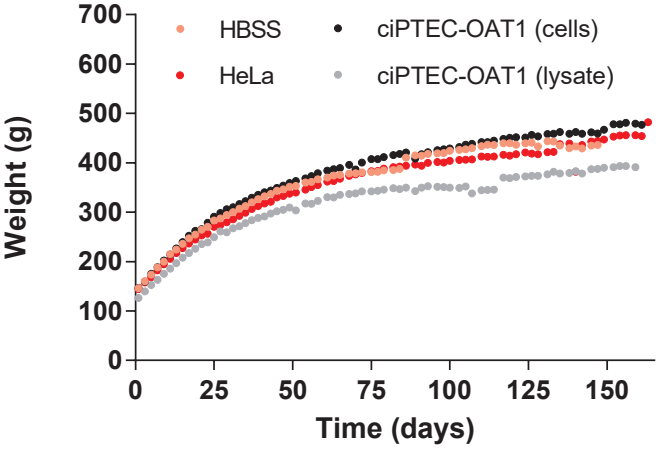


Figure 6.S4. Weight measurements of rats during 5 months study. Weight measurements (grams; g) in (**a**) male and (**b**) female rats during the follow-up. No big differences in weight maintenance were observed for any of the treatment groups in both sexes.

CHAPTER VII

GENERAL DISCUSSION

GENERAL DISCUSSION

The kidneys play a crucial role in maintaining body homeostasis by regulating blood volume and blood pressure, electrolyte balance, and pH, and by exerting endocrine functions in terms of producing several hormones such as erythropoietin, 1α,25-dihydroxy-vitamin D_3 and renin [1-7]. The role in filtering blood and eliminating xenobiotics like drug metabolites and endogenously produced and food digestion-derived compounds is also extremely important [8,9]. Various kidney diseases can result in the reduction of these important functions. For patients with end-stage renal disease (ESRD), renal replacement therapy such as kidney transplantation or dialysis are available [10,11]. The former one, even though being preferred as a treatment modality, is limited by shortage of organ donors and complicated immunosuppressive therapies [12]. Hemodialysis and peritoneal dialysis on the other hand, offer only a limited amount of filtration and lack metabolic and endocrine functions. Consequently, dialysis is inferior to transplantation in terms of patient survival and quality of life, and is also more costly. [13,14]. Novel treatment options could be beneficial if they are able to replace regulatory, metabolic and endocrine kidney functions in addition to filtration. Even though innovative treatment modalities developed in recent years were proven to be effective, their actual clinical application is still far from reality. In addition to technical and manufacturing hurdles, there are safety issues related to the advanced cell-based products that require a thorough preclinical evaluation [15-17].

Do ciPTECs meet essential requirements for BAK application?

During recent years much effort has been devoted to develop a bioartificial kidney (BAK), consisting of a scaffold seeded with a certain cell type. In order to be suitable for BAK application, a particular cell type should be able to replace kidney functions that are not replaced by dialysis, such as endocrine and metabolic activities and notably active transport of waste metabolites, such as protein-bound uremic toxins [18]. Many cell types used previously and examined for BAK use, such as animal-derived MDCK and LLC-PK_1 cells, showed limited transport function [19,20]. Primary PTEC would represent the most relevant cell model from a physiological point of view [18]. However, even though their use in Renal Epithelial Cell System (BRECS) showed the presence of aminopeptidase-N and Zonula Occludens 1 (ZO-1), as well as oxygen consumption and glutathione metabolism as indicators of cell metabolic activity, the vitamin D activation as evidence of endocrine activity and the presence of fundamental PTEC transporters have not been demonstrated [21,22]. On the other hand, ciPTECs do possess the required set of essential transporters responsible for uptake and removal of uremic solutes. The expression of most of them, including OCT2, P-gp, BCRP and MRP4, has been previously shown [23-27]. The lentivirally-mediated overexpression of OAT1 and OAT3 is a significant addition to the cell model as the expression of these transport proteins is rapidly lost in PTEC during culture [28,29]. The expression of these transporters in ciPTECs potentially allows the removal of many uremic waste molecules, which is a prerequisite for BAK application. This was best shown in a 3D environment when cells were cultured on biofunctionalized polyethersulfone membranes and were able to mediate the transepithelial secretion of protein-bound uremic solutes, such as indoxyl sulfate and

kynurenic acid [25]. Furthermore, the ability of ciPTECs to form tight and polarized epithelial monolayers (Chapter 3), as well as protein reabsorption through receptor binding and subsequent endocytosis (Chapter 6) are other key features present in ciPTECs [25,30]. Moreover, for the first time, ciPTECs were shown to be able to mediate synthesis of 1α,25-dihydroxy-vitamin D_3, starting from 25-hydroxy-vitamin D_3 by 1α-hydroxylase (Chapter 3). In addition to being a prerequisite for future BAK use, the most active form of vitamin D has protective effects on ciPTEC viability, inflammatory and oxidative status, with subsequent improvement of transepithelial barrier function, as was previously shown for other cell types and organs [31-35]. Accordingly, ciPTECs seem to be a valid choice for BAK application as far as it concerns essential endocrine, metabolic and transport activities [24,25,36-38]. Finally, cells intended for BAK use have to be readily available to meet clinical needs, and while this can pose a significant limitation to the use of primary cells, ciPTECs could help overcome this barrier in BAK development. This is particularly evidenced by ciPTECs' long lifespan and compatibility with upscaling BAK approach.

How to overcome safety hurdles in BAK development?

Development and clinical application of cell-based products require extensive examination of risk factors and safety issues linked to both scaffold and cell components. In the work presented in this thesis, attention was given to the cell constituent of BAK. According to the systematic review presented in Chapter 2, in the context of kidney disease, consistent safety evaluation of various genetically modified cell-based therapies is still missing, or is addressed under suboptimal or even inappropriate study designs. In addition, due to the large heterogeneity of studies evaluated, especially regarding animal and disease models, cell type, cell number and administration route, as well as type of genetic manipulation, it was extremely difficult to gather the required information for correct evaluation of safety of cell therapies for renal diseases. For instance, even though many studies employed stem cells with various degrees of differentiation potential, concerns related to that issue such as transdifferentiation ability, distribution in non-target tissues and even tumor or teratoma formation, were scarcely evaluated. Based on the findings described in Chapter 2, it is clear that there is a need to perform further, and above all, well-designed preclinical studies in order to get more insight into the possible risks associated to cell therapy for kidney disease. This information should facilitate implementation of cell-based therapies into clinical applications, for which there are various official guidelines and recommendations offered by world leading health and medicinal regulatory organs and agencies, including World Health Organization (WHO), U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) [15-17].

Immunogenic and inflammatory effects of ciPTECs

Moving on to BAK development and possible use of ciPTECs, several key aspects of preclinical safety evaluation were examined in this thesis. The potential immunogenic effect of ciPTECs that are of allogeneic origin, was presented in Chapter 4. A thorough characterization, under various CKD-relevant conditions, showed that ciPTECs do not express high levels or in some cases even lack the expression of immunogenic surface molecules, including Human Leukocyte Antigen (HLA) molecules from class I and II (HLA-A, -B, -C, -DR), CD40, CD80, and CD86. Although

able to mediate an inflammatory response by the release of several cytokines (Interleukin (IL) -6, 8 and Tumor Necrosis Factor-α (TNF-α)), the cells were not able to induce activation and proliferation of immune cells, such as T lymphocytes *in vitro*. This underlines a relative safety of ciPTECs regarding systemic immunotoxic effects. However, as suggested by the official guidelines, further evaluation in an *in vivo* setting would be required to evaluate more specifically both humoral and cell-mediated responses [39]. Since the intended use of ciPTECs in BAK is an extracorporeal application, in which ciPTECs should be separated form the circulation by a membrane, it would be more relevant to examine the effects mediated by secreted soluble factors, rather than the consequences of direct contact and interaction with immune cells. Findings presented in Chapter 5 show that when cultured on biofunctionalized hollow fiber membranes and in the presence of inflammatory stimuli, ciPTECs are able to secrete IL-6 and IL-8 in a polarized manner. In particular, cytokines were predominantly secreted to the apical compartment compared to the basolateral which, considering a BAK design as presented in Figure 7.1, would imply that the amount of such mediators released in the circulation might not pass threshold levels. The same behavior was observed for other immunomodulators such as soluble forms of HLA molecules. This, additionally, suggests that the safety of cell-based therapeutics has to be judged in the context of device architecture and mode of application. For instance, multiple treatment sessions of an animal model of ESRD with a BAK device could help confirm *in vitro* findings and evaluate whether there are any effects of polarized secretion of immunomodulators.

Differentiation, genetic stability and tumorigenic effect of ciPTECs

Previous studies and work presented in this thesis (Chapter 6) offer data on the differentiation, genetic stability and tumorigenicity of ciPTECs. Upon conditional immortalization, ciPTECs were characterized for differentiation status and expression of specific markers in relation to SV40T expression. Namely, it was shown that after 7 days of culturing at non-permissive temperature for SV40T, its expression goes down and specific PTEC markers are expressed, including a marker of epithelial monolayers, tight junction protein ZO-1, a specific brush-border membrane enzyme, aminopeptidase N (CD13), and several PTEC-specific transporters, including aquaporin 1 (AQP1), MRP4, BCRP, P-gp, and OCT2 [23,30]. The work presented in Chapter 6 shows the ability of cells to proliferate in a controlled manner, meaning that they do not grow in the absence of anchorage and remain sensitive for contact inhibition, regardless of SV40T expression. Even though we could not demonstrate that the integration of transgenes causes any insertional mutagenesis events with functional alterations or oncogenes activation, the cells do present an abnormal karyotype, which progresses into complex chromosomal aberrations after prolonged culture. This is most likely due to SV40T effects on the spindle assembly checkpoint protein Bub-1 [40]. However, this did not result in functional alterations of ciPTECs as far as it concerns BAK-related tasks, including transport activity and vitamin D activation. To examine whether the abnormal karyotype could affect tumorigenicity of ciPTECs, an *in vivo* study was performed using athymic nude rats and cells at a passage number slightly below the one presenting the highest degree of chromosomal alterations. This study provided no evidence for the presence of ciPTEC tumorigenic and oncogenic effects, as described in Chapter 6. Considering recommendations of

regulatory agencies (WHO, FDA, EMA), a stable, diploid karyotype is preferred for cells intended for therapeutic use, as it reduces the risks associated with sudden changes in cell function and behavior due to chromosomal aberrations [15]. The absence of tumorigenic effect *in vivo* and a lack of altered cell function of ciPTECs argue for their safe use in BAK.

Ultimately, if diploid, stable cells are absolutely required, for direct administration or implantable devices for instance, alternative approaches should be implemented to re-engineer or re-create cells and make them genetically stable. A great promise in this respect is the breakthrough genome editing technology, CRISPR-Cas9 system. With this technique cells can be modified in a precise manner, while maintaining genetic integrity [41,42]. This allows creation of cells for therapeutic application, without using viral vectors that could cause genome damage [43]. However, also this solution has to be approached very carefully, as a recent study suggested that CRISPR-Cas9 editing might be able to induce unexpected, off-target mutations [44,45].

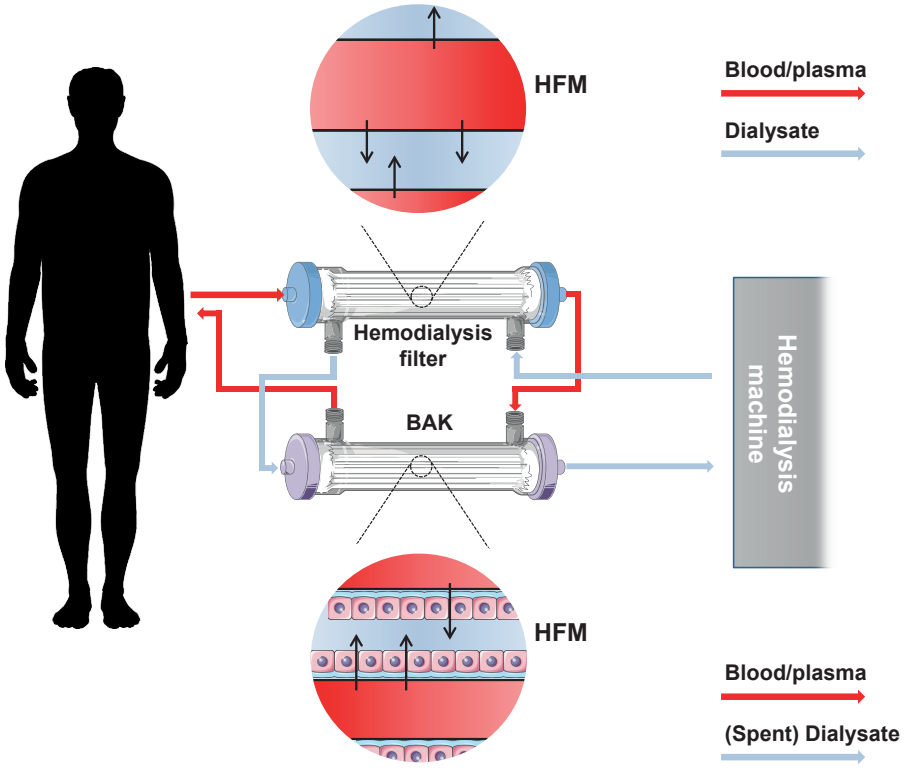


Figure 7.1. Schematic representation of BAK device and proposed mode of application. Hemodialysis filter incorporated in series with ciPTEC-containing BAK in an extracorporeal blood perfusion circuit. CiPTECs grown inside the hollow fiber membranes (HFM) can actively transport uremic toxins from the blood/plasma (basolateral compartment, inter-fiber space) into the (spent) dialysate (apical compartment; fiber lumen).

Retroviral vectors as a safety concern

When cells are modified via retroviral and/or lentiviral vectors, two essential safety matters have to be addressed. Firstly, there could be events of insertional mutagenesis and altered expression of endogenous genes due to a random genomic integration of the viral vectors [46,47]. This was addressed in Chapter 6, where the genomic locations of the transduced genes were evaluated as well as the number of copies of the transgenes that were integrated in ciPTECs. Most importantly, we did not find alterations in endogenous genes. Secondly, when retroviral vectors are used to stably express transgenes in the cells, there is a possibility for production of replication-competent retro- or lentiviruses (RCR, RCL) [48]. Even though replication-incompetent viral vectors are used they can regain the deleted genes required for replication through unforeseen recombination processes. The main feared consequence of this phenomenon is the transmission of oncogenes. The presence of RCR and RCL can be assessed by adding cell culture supernatants to highly permissive cell lines followed by PCR techniques to detect viral specific components [15,49]. Considering that ciPTECs were created with viral vectors bearing transgenes with oncogenic activity (hTERT and SV40T), RCR and RCL events could have far-reaching negative consequences. Therefore, it is extremely important to evaluate whether there are replicating viruses released by ciPTECs. Previous unpublished results of product-enhanced reverse transcriptase (PERT) assay confirmed that ciPTECs do not produce any replication competent viruses that could be spread in the environment (the activity of reverse transcriptase (RT) detected was 0 nU/ml). However, if cells are considered for clinical use, this analysis should be done regularly and particularly at the final stages of production as to ensure risk-free application.

Animal-derived products and related risks

All cell types, including those being characterized and developed for therapeutic purposes, are grown and cultured in specific culture media containing a wide range of compounds and nutrients, including glucose, amino acids, vitamins etc. However, very often such enriched culture medium is not sufficient and additional components such as growth factors and hormones have to be added. Common practice is the use of fetal bovine serum (FBS) at 5-10% (v/v) to supplement the medium with additional growth factors in order to allow optimal cell attachment, growth and proliferation. Even though it has been in use for more than 50 years, FBS has never been completely characterized and the composition is subject to considerable batch-to-batch variability [50]. Moreover, the use of animal-derived serum can impose biosafety risks, notably by cell contamination with infectious agents such as bacteria, fungi, mycoplasms and viruses [51,52]. In particular, bovine serum can be contaminated by various viruses, including Bovine viral diarrhea virus (BVDV) and polyomavirus [15,53]. Since serum is usually produced by making large pools derived from up to thousand animals, only one infected animal can cause contamination of the entire pool. Therefore, a thorough and regular screening of animal-derived serum should be performed by various assays, including hemadsorption assays, immunofluorescence for specific viruses, and cytological stainings using cells that are permissive for a wide range of bovine viruses, such as MDCK or Vero cells [15]. In addition, it should be proven that risk-containment strategies regularly applied, such as inactivation by heat or irradiation, are sufficient to effectively inactivate

microorganisms that were not inactivated during the manufacturing and quality control of the serum. For instance, if radiation is used a specific dose should be applied in order to avoid the destruction of biological properties of the components, while reducing the contamination risk. Based on these considerations and also taking the ethical aspects associated with FBS harvesting into account, a complete transition to synthetic media would be preferred [54-56].

Culturing cells in a medium without serum supplementation could greatly reduce the risk of microbiological contaminations and endotoxins, and on the other hand allow for better, chemically-defined culturing conditions thus improving cell performance both for therapeutic applications and research [50]. An attractive option is the use of serum-free media supplemented with plant constituents (eg. Prolifix) containing molecules that can replace mitogenic molecules present in animal serum [50]. An example is given by *in vitro* fertilization (IVF) procedures where human embryos are cultured in the presence of recombinant human albumin to minimize and avoid any risk associated with viral or other contaminations [50,57].

However, it should be noted that every cell type has its own requirements for optimal growth and proliferation, hence it appears extremely difficult to develop a universal serum-free medium [55]. Therefore, a cell-specific growth medium could be developed with addition of various singular components, including purified or recombinant hormones, growth factors, protease inhibitors, proteins, amino acids, glutamine, lipids, and trace elements, as previously shown for the enterocyte cell line Caco-2 [58]. Then, the cells should undergo a gradual weaning process to allow progressive adaptation to low serum concentrations until reaching serum-free conditions. Finally, cells should be evaluated again for specific marker expression and performance, as an unwanted selection and subsequent change in cell population could have occurred during the adaptation procedure [55].

As for ciPTECs, a similar procedure could be applied to culture the cells in serum-free conditions in order to further improve their safety profile as well as to restrict batch-to-batch variability. Preliminary data of ciPTECs cultured in serum-free medium for 24 h and 48 h showed only a slight drop in cell viability of approximately 10% (Figure 7.2).

It should be noted that the serum-free medium in which cell viability was tested contained all supplements (insulin, transferrin, selenium, triiodothyronine, epidermal growth factor and hydrocortisone) that are also added to the regular serum-containing medium, suggesting that cells can be grown in serum-free culture medium provided that the necessary growth factors and components are present. Therefore, if a gradual process of weaning is done correctly, ciPTECs could eventually reach serum-free conditions without harnessing the viability. This would avoid the risks associated with animal-derived materials.

Scaffold-related safety issues

In addition to the cells, the biomaterial or scaffold used as support for the cells should be biocompatible. The currently used polymer to grow ciPTECs for BAK application is microPES® TF10 hollow fiber capillary membrane produced by solvent induced phase separation from polyethersulfone (PES) with added polyvinylpyrrolidone (PVP) to create a balance between wettability and biocompatibility. This type of membrane is shown to be hemocompatible and

biocompatible, considering its current use in plasmapheresis and blood collection for donation [59]. On the other hand, it also has to be cytocompatible for ciPTEC culture. Given that the surface properties of microPES® hollow fibers do not allow cell attachment, a coating with L-3,4-dihydroxyphenylalanine (L-DOPA) and collagen IV was necessary to obtain fully cytocompatible and biofunctionalized membranes [25,38]. However, the composition, molecular weight cut-off and coating components could influence the passage of nutrients from the circulation to the cells and the transfer of soluble factors released by the cells to the circulation. The currently used membranes have a wall thickness of 100 µm, an inner diameter of 300 µm and maximal pore size of 0.5 µm. A previous study [38] and also Chapter 5 partly examined the effects of double coating on pore size by measuring passage of proteins (bovine serum albumin and IgG) and water permeance. Collectively, the results show that coating can slightly decrease the membrane pore size but it does not alter water permeance and proteins passage, suggesting that the physiological function i.e. transport of nutrients and toxins is maintained [38]. Moreover, the fact that albumin (approximately 66 kDa) can pass the membrane means that it allows the removal of protein-bound toxins by bringing them in close proximity to the cells. The final barrier to the passage, and eventual undesired loss, of proteins is the strong epithelial monolayer that is formed by the ciPTECs. In addition, the ciPTEC monolayer also serves as a barrier to the passage of apically released soluble molecules such as cytokines, as presented in Chapter 5. Finally, the presence of a flow in the BAK device during its clinical application should enable faster passage and removal of such apically secreted mediators, thus reducing further the risk of entering the patients' blood circulation.

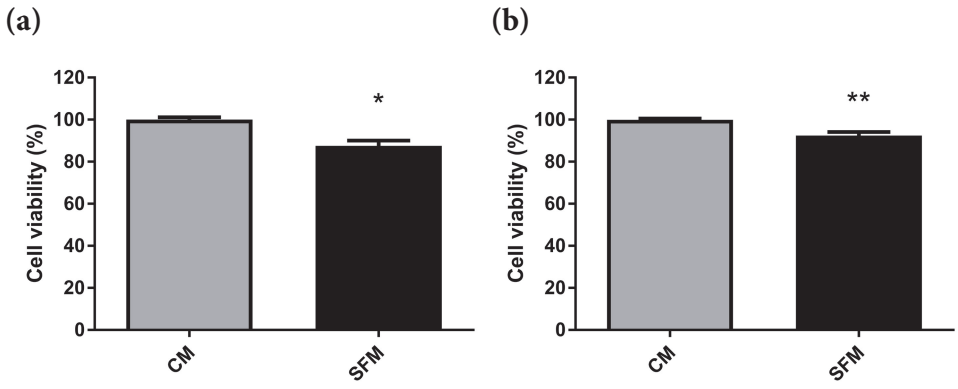


Figure 7.2. CiPTEC viability in serum-free medium. CiPTEC viability after (**a**) 24 h and (**b**) 48 h exposure to serum-free medium (SFM) compared to the complete medium (CM) containing 10% fetal bovine serum (FBS). Both CM and SFM contain supplements necessary for the growth of proximal tubule epithelial cells: insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 µg/ml), triiodothyronine (40 pg/ml), epidermal growth factor (10 ng/ml) and hydrocortisone (36 ng/ml). *p<0.05, **p<0.01 (SFM compared to CM; unpaired t test)

An additional safety issue related to the biomaterial component of BAK is the coating used to support cells. For instance, L-DOPA, that is present in polymerized form on the fibers to allow cell attachment, is a precursor of dopamine, a catecholamine and neurotransmitter with a variety of biological functions [60]. Therefore, possible degradation of the polymer form of L-DOPA and its release over time under BAK-relevant conditions should be measured to rule out any adverse effect.

Finally, alternative extracellular matrix (ECM) components could be further explored and considered for efficient cell growth. As indicated previously, ECM consists of various types of macromolecules, such as proteoglycans and fibrous proteins that support cell adhesion and proliferation [61]. The most important proteoglycans in ECM are chondroitin sulfate, heparan sulfate, keratin sulfate, hyaluronic acid, while the main fibrous ECM proteins are collagen, elastin, laminin and fibronectin [61,62]. Considering the ability of various fibrous ECM components to influence cell attachment, proliferation, differentiation and migration it is clear that appropriate ECM coating of biomaterials can improve cell function, despite the fact that cells can also secrete their own ECM molecules [23]. The optimal coatings for renal cell growth, as shown by monolayer formation and ZO-1 expression by the HK-2 cell line, are laminin, gelatin, Matrigel®, collagen IV and L-DOPA, while pronectin, collagen I, poly-L-lysine and poly-D-lysine are less suitable [63-65].

Perspectives on cell-friendly BAK environment for final safety assessments

Finally, the BAK device composition and structure should be known in order to complete the safety profile. In Chapter 5 we presented some encouraging findings related to the polarized secretion of immune modulators. However, the final BAK device, suitable for kidney patients, should contain far more fibers and cells in order to achieve the required clearance of toxins. Preliminary calculations based on single hollow fiber transport experiments suggested that using a flow rate of 200 ml/min and a total membrane surface of 1.8 m^2 it would take approximately 30 min to clear indoxyl sulfate and kynurenic acid to reach normal plasma concentrations [66]. Taking into account these parameters and the design of the device presented in Chapter 5, this would mean that no less than 135 fibers with a length of 8.5 cm^2, are needed to achieve a similar clearance. This may even be an underestimation since many solutes, uremic toxins, and drugs can compete for the same transporters [8,67-70]. Therefore, research should be aimed at developing a device with improved configuration that can contain a sufficient number of hollow fiber membranes and cells without altering the desired functional aspects. Moreover, instead of evaluating the clearance of isolated, single toxins, it would be more appropriate to use either a specifically designed mixture of multiple compounds or even plasma samples derived from uremic patients. In this way, based on the preferred configuration of the device, it would be possible to predict and determine the exact clearance of protein-bound uremic toxins. For instance, this could be achieved by developing mathematical and computational models of kinetics of uremic toxins that account for dynamic equilibrium between protein, toxin and the protein-toxin complex, as suggested previously [71].

From the BAK engineering point of view, several safety issues should be addressed. First of all, it is necessary to examine how the anticipated use of the device can affect ciPTECs. Based on the device configuration and application, ciPTECs could be exposed to dialysate solution, to spent dialysate (containing waste to be eliminated) derived from a hemodialysis filter connected in series with the BAK, and to human plasma (Figure 7.1). Considering all factors that are present in (spent) dialysate and plasma, including uremic waste molecules, drugs and many other compounds, ciPTEC viability would be the first parameter to assess, as unforeseen and undesired cell death could alter the efficacy of BAK but also increase significantly the safety concerns because cell death could lead to the release of various bioactive intracellular components. Preliminary results regarding the effect of human plasma derived from uremic patients on ciPTEC viability, as depicted in Figure 7.3, show that 24 h exposure to high concentrations of human plasma (80%) derived from CKD patients significantly reduced cell viability, while lower concentrations (20%) did not seem to be very toxic (approximately 10-15% reduction in cell viability). This finding gives a clear indication of what could be expected in BAK environment even though shorter exposure periods could be tested, as BAK treatment ideally should not exceed the current 4 h duration of a hemodialysis session.

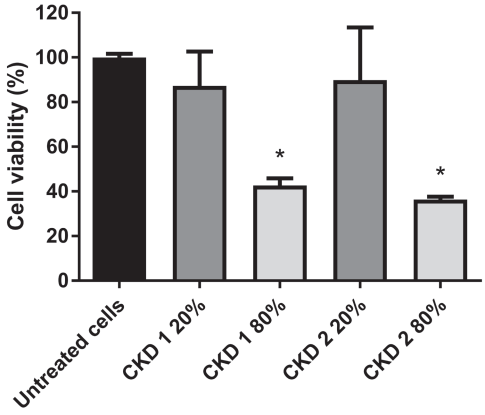


Figure 7.3. Effect of CKD plasma on ciPTEC viability. CiPTEC viability after 24 h exposure to plasma (20% and 80%) derived from CKD patients. CKD1 – mild CKD (creatinine 300 µmol/l); CKD2 – severe CKD (creatinine 530 µmol/l; eGFR < 20 ml/min/1.73m^2). *p<0.05 (compared to untreated cells; One-way ANOVA, Dunnett's multiple comparison test)

In addition to cell viability, the potential adverse response of cells to the presence of plasma, in terms of altered cell transport activity, should be investigated. Moreover, other factors related to the final BAK design which could possibly influence cell function and viability, such as the presence of flow, appropriate nutrients and oxygen, should be addressed. Indeed, it has been

suggested previously that culturing primary PTEC in a microfluidic device and in the presence of flow conditions and shear stress of 0.2 dyne/cm^2 can enhance epithelial polarization and mechanosensing by increasing expression of primary cilia. This was associated with improved albumin transport, glucose reabsorption, and efflux P-gp transporter activity [72,73].

With the aim to establish an attractive new therapeutic solution for ESRD patients, represented by the ciPTEC-containing BAK device for the efficient removal of uremic waste molecules, compelling evidence has been obtained both in terms of function and safety. Final efforts are required to optimize the current design of the device and to model and predict the clearance of uremic toxins. Remaining biosafety points should be addressed preferably in *in vivo* models of uremic syndrome, which could allow the translation of the results obtained *in vitro* to a more complex and physiologically relevant system. The future research will be focused on the development of the final device and evaluation of its efficacy and safety in a CKD animal model (nephrectomized rat) after multiple treatment sessions. To determine the clinical relevance of the BAK, the system is going to be tested both as an individual treatment as well as in combination with a standard hemodialysis device, applied in series. Besides the evaluation of clearance capacity regarding uremic toxins, safety aspects specifically related to the immune system activation and toxicological effects (the effect of human uremic plasma and dialysate solution on ciPTEC viability) are going to be determined.

The road of BAK to the market: perspectives and challenges

The standard development procedure of advanced therapy medicinal products (ATMPs) (Figure 7.4), such as somatic cell therapy and tissue-engineered products, is a long and complex process. The development encompasses (1) the basic research and experimental observations, (2) a preclinical phase with animal testing under Good Laboratory Practice (GLP), (3) clinical trials under Good Clinical Practice (GCP), Good Manufacturing Practice (GMP) and Good Pharmacovigilance Practice (GVP), (4) authorization, registration and marketing, which is finally followed by (5) postauthorization, focusing on pharmacovigilance with the scope to monitor, assess and prevent any side effects or adverse reactions associated with the medicines and therapeutic products [74,75]. The EMA has established a committee, The Committee for Advanced Therapies (CAT), responsible for assessing the quality, safety and efficacy of ATMPs prior to marketing authorization [76,77].

After a thorough basic research regarding ciPTEC characterization, function and ability to perform required tasks for improved ESRD treatment (active secretion of uremic waste metabolites and vitamin D activation), the development of the ciPTEC-based BAK device is currently in the preclinical stage. In addition to the work presented in this thesis, which is mainly focused on the safety aspects of ciPTEC line, future work will focus on confirming the safety and efficacy of BAK in a relevant animal model of ESRD (nephrectomized rat) to provide enough information for ethical committees and regulatory agencies to decide for further development and eventual clinical trials. However, several aspects have yet to be determined in order to continue with further development of the BAK. One of the critical questions that has to be addressed in the future preclinical studies is the most effective and safe mode of application of the BAK. It has to be

determined whether it will be perfused with blood or plasma following plasmafiltration procedure, and whether the device could be re-used or not. The latter would mostly depend on cell viability and functional recovery after a single treatment session. In addition, various manufacturing-related topics should be examined in the current phase of development. For instance, prior to clinical testing, the manufacturing process should be determined in order to ensure consistent, reproducible and high-quality final product for safe use in patients. Regarding this issue, the mode of storage and shelf-life of the final product will have to be established. In particular, the optimal cryopreservation conditions have to be determined in order to ensure a safe and functional device with viable cells after thawing and reconstitution. This is an extremely important point to evaluate as it might affect the manufacturing procedures and future supply chain strategies [78]. The possibility to develop and store a safe and high-qaulity device would allow the production of an off-the-shelf product which could be manufactured in large-scale manufacturing facilities, in a stable and standardized manner, from where it could be distributed to specialized medical centers. Nonetheless, very careful transport conditions woud have to be ensured in order to avoid any damage of the final product.

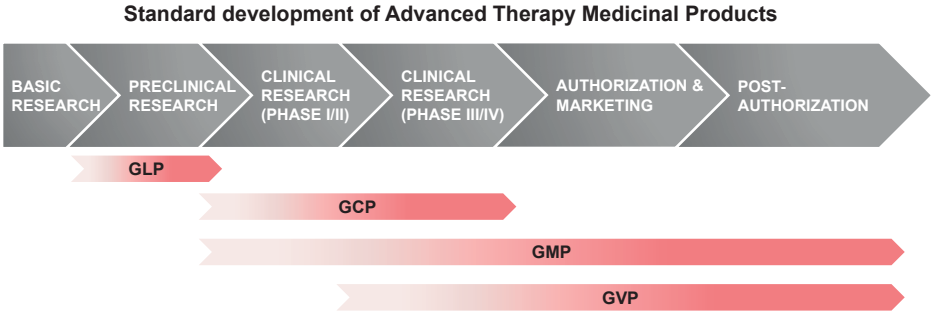


Figure 7.4. Stages of standard development of Advanced Therapy Medicinal Products (ATMPs). The first phase is experimental observations which is performed to study the ingredients of the medicinal product, in particular cells, and to establish the mechanism of action and relevant functions. In the preclinical phase, efficacy and safety of cells are examined *in vitro* and in *in vivo* relevant models. Clinical trials consist of Phase I (safety, dosage and side effects evaluation), Phase II (initial clinical research on treatment effect for a given medical condition or disease), Phase III (safety and efficacy evaluation in conditions of common use and compared to currently available therapeutic alternatives) and Phase IV (pharmacovigilance). Following clinical research, the device undergoes the authorization, registration and marketing. Finally, the postauthorization phase is based on pharmacovigilance and it serves for the assessment, awareness and prevention of possible adverse effects related to the use of the ATMP. GLP - Good Laboratory Practice; GCP - Good Clinical Practice; GMP - Good Manufacturing Practice; GVP - Good Pharmacovigilance Practice. Adapted from [75].

Finally, it should be noted that the manufacturing costs, market size, risk/benefit pofile and multiple applications will influence the price of the device and/or treatment sessions, which is an extremely important challenge that advanced therapies are facing nowadays [78].

In conclusion, given the genetically modified nature of ciPTECs, the currently considered and proposed BAK application is extracorporeal rather than implantable. Nevertheless, the safety evaluation of ciPTECs, as presented in this thesis, offers a significant contribution to the preclinical development and evaluation of the BAK device and provides further advances in the field of innovative renal replacement therapies.

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CHAPTER VIII

SUMMARY

NEDERLANDSE SAMENVATTING

SAŽETAK NA SRPSKOM

LIST OF ABBREVIATIONS

SUMMARY

A next step towards bioartificial kidney: preclinical safety evaluation

Our kidneys play an important role in various processes necessary for normal body function and homeostasis, including regulation of water and electrolytes, acid-base balance, blood pressure, as well as endocrine and metabolic functions, and removal of metabolic waste products and xenobiotics. Therefore, it is clear that kidney disease and loss of kidney function can give rise to severe clinical manifestations. Currently available treatment options for patients affected by chronic kidney disease (CKD) comprise dialysis (peritoneal and hemodialysis) and kidney transplantation. However, as there is a significant shortage in organ donors available for kidney transplantation and dialysis treatment does not offer complete removal of uremic waste metabolites, especially protein-bound molecules, several novel treatment options are being developed. These should improve clinical outcome and overall well-being of CKD patients. A promising solution may be offered by the bioartificial kidney (BAK), a device that combines novel biotechnological and tissue engineering approaches used for the development of advanced medicinal therapy products. BAK aims to replace the loss of kidney function observed in CKD, by combining a scaffold such as polymer hollow fiber membranes (HFM) and renal proximal tubule epithelial cells (PTEC), thus creating biofunctionalized kidney tubules that would help replacing not only excretory, but also endocrine, metabolic and regulatory functions of the kidney. These features are currently absent in standard dialysis treatment.

One of the attractive options for the use in renal replacement therapies are the conditionally immortalized human PTEC (ciPTEC). The ciPTECs were created by immortalizing primary PTEC via retroviral transductions with a temperature-sensitive mutant of Simian virus 40 Large T antigen (SV40T), U19tsA58, and with the catalytic subunit of human telomerase (hTERT). This allows continuous expansion and availability of cells, which helps overcoming a lack of sources usually observed when primary cells are used in tissue engineering applications. In addition, since these cells are equipped with some of the most important transporter proteins, including among others, organic anion transporters 1 and 3 (OAT1/3), organic cation transporter 2 (OCT2), breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), multidrug and toxin extrusion proteins 1 and 2 (MATE1/2) and multidrug resistance proteins 2 and 4 (MRP2/4), responsible for handling of various waste products, it is not unexpected that ciPTECs represent one of the most obvious choices for applications such as BAK.

Despite the promising features of ciPTECs for use in BAK, the safety issues such as immune response activation, tumorigenic potential and other undesired effects, should be carefully evaluated in a preclinical setting.

The present thesis describes the preclinical safety evaluation of ciPTECs for BAK application. **Chapter 2** gives a literature overview concerning the use of genetically modified cells in animal models of kidney disease for the purposes of cell therapy. The relevant scientific publications were collected using a systematic literature search in PubMed and EMBASE databases. The articles that met the pre-defined inclusion and exclusion criteria were further analyzed and used to determine the safety of genetically modified cells in the treatment of kidney disease. It appeared that in

general, cell therapy for kidney disease did not impose a significant risk, as far as it concerned tumorigenic and teratogenic effects. However, due to the high heterogeneity in study characteristics (animal species, disease model and cell therapy) and the fact that the majority of the studies were characterized by suboptimal or inappropriate study design, it was rather challenging to reveal a general overview on safety aspects. Therefore, further research on cell-based therapies for kidney disease, with well-designed preclinical studies is strongly encouraged.

Beside the proven uremic toxin transport activity, ciPTEC-containing BAK could benefit from endocrine functions that are lost in kidney disease and not restored by dialysis therapy. The focus of **Chapter 3** was to evaluate the ability of ciPTECs to activate and secrete the biologically most active form of vitamin D, 1α,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$) also known as calcitriol, starting from its precursor 25-hydroxy-vitamin D_3 ($25(OH)D_3$). The main findings were that ciPTECs were able to activate the vitamin D in 1α-hydroxylase-dependent manner, and that this reaction was not affected by uremic conditions typical of kidney patients. In addition, vitamin D can exert beneficial effects on ciPTECs in uremic conditions in terms of restoration of cell viability, improved inflammatory and oxidative status of the cells, as well as recovery and reinforcement of transepithelial barrier function. All these aspects can be of great asset to proper BAK function.

Chapter 4 describes the allostimulatory capacity of ciPTECs, addressing the immunogenicity-related safety concerns of a cell therapy. Kidney- and urine-derived ciPTECs were extensively characterized under a variety of stimulatory conditions for the expression and release of most relevant molecules for the activation of an alloimmune response. Even though both ciPTEC lines were shown to express CD40 and HLA class I molecules with an increase after exposure to interferon-γ (IFN-γ) and bacterial lipopolysaccharide (LPS), the expression of HLA class II molecules, and the costimulatory molecules CD80 and CD86 was very low or completely absent. In addition, ciPTECs were also shown to lack direct allostimulatory effects as they failed to induce activation and proliferation of human peripheral blood mononuclear cells (PBMCs) *in vitro*. Finally, BAK-relevant conditions in which ciPTECs are separated from PBMCs by a biofunctionalized (L-DOPA and collagen IV coated) polyethersulfone membrane, revealed that even strong inflammatory conditions (LPS) or uremic environment (plasma from kidney disease patients) did not alter ciPTEC's transepithelial barrier, as measured by FITC-inulin diffusion.

Immunogenicity-related safety of ciPTECs investigated in chapter 4, was further probed in **Chapter 5**. Here, the release of proinflammatory and immune mediators including interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α) and soluble HLA class I (sHLA-I) molecules in an upscaled BAK device containing multiple HFM with ciPTECs, was investigated. The results show that there is a fully polarized secretion of the mentioned mediators, mainly oriented towards the apical side, or dialysate compartment, and not towards the basolateral side that would correspond to the side of the patient's body fluid. In addition, monolayer formation, transepithelial barrier integrity and transepithelial transport of indoxyl sulfate were demonstrated as well. Altogehter, these results indicate a successful upscaling of BAK device with functional ciPTEC epithelial monolayers and polarized secretion of inflammatory mediators for safe future applications.

The potential tumorigenic and oncogenic effects of ciPTECs are investigated in **Chapter 6**. Hereto, a series of *in vitro* experiments examined the features of ciPTECs at permissive (33 °C) and non-permissive (37 °C) temperatures, given the nature of SV40T's mutant expression and function. The results show that SV40T expression is greatly reduced at 37 °C starting from 1 day of incubation. Regarding proliferation rate and cell cycle distribution, cells were shown to behave accordingly to the temperature-dependent expression of SV40T, with higher proliferation at permissive temperature. Moreover, cells at non-permissive temperature appeared sensitive to p53 mediated-apoptosis. Cell growth did not occur in the absence of anchorage and it did obey the phenomenon of contact-inhibition. Genetic stability was assessed by karyotyping and genomic integrations of transgenes, which showed that the expression and function of endogenous genes are not affected (eg. endocytosis capacity). Despite minor events of cell invasion through an extracellular matrix and unstable karyotype upon prolonged cell culture, *in vivo* experiments showed the absence of tumorigenic or oncogenic effects of ciPTECs when injected in immunodeficient rats. Based on the obtained results ciPTECs appear to be rather safe option for future BAK applications.

Finally **Chapter 7** gives an in-depth discussion of the obtained results as well as suggestions and perspectives on further BAK development. The results on ciPTECs' safety presented in this thesis, including absence of immunogenic and tumorigenic effects and presence of desired endocrine functions, offer a valuable addition to the ciPTEC-based BAK development and encourage further preclinical testing in uremic animals, a prerequisite for future clinical trials.

NEDERLANDSE SAMENVATTING

Een nieuwe stap op weg naar de biologische kunstnier: preklinisch veiligheidsonderzoek

Onze nieren zijn verantwoordelijk voor het handhaven van het interne milieu van het lichaam, o.a. door het reguleren van de water- en zoutbalans en de bloedruk, de productie van hormonen en de verwijdering van afvalproducten uit het bloed. Het is duidelijk dat nierziekten of verlies van nierfunctie gepaard gaan met ernstige gezondheidsproblemen. Bij een chronische nierziekte is er vaak een geleidelijke verdere afname van de nierfunctie met uiteindelijk eindstadium-nierfalen waarvoor momenteel alleen dialyse en niertransplantatie de behandelingsmogelijkheden zijn. Mede door het tekort aan beschikbare donororganen voor niertransplantatie zijn veel patienten afhankelijk van dialyse. Dialyse biedt echter maar beperkte mogelijkheden om afvalstoffen uit het bloed te verwijderen en er is daarom behoefte aan nieuwe behandelstrategieën. Deze zouden de gezondheidsproblemen moeten tegengaan en het algehele welbevinden van de patient moeten verbeteren. De biologische kunstnier (BAK) zou hiervoor een oplossing kunnen bieden. In zo'n kunstnier worden nieuwe biotechnologische processen gecombineerd met weefselontwikkeling om tot een geavanceerd biomedisch product te komen dat de orgaanfunctie (deels) kan vervangen. De BAK heeft tot doel om het verlies in nierfunctie te vervangen door gebiofabriceerde nierbuisjes, holle buisjes die bekleed zijn met menselijke niertubuluscellen. Deze buisjes zouden niet alleen verantwoordelijk moeten zijn voor de uitscheiding van afvalstoffen, maar ook de hormonale, metabole en regulatoire functies van de nier moeten kunnen nabootsen. Deze functies zijn momenteel niet aanwezig in de dialysebehandeling.

De conditioneel-geïmmortaliseerde niertubulusepitheelcel (ciPTEC) is een aantrekkelijk celmodel om in nierfunctievervangende therapieën, zoals de BAK, toe te passen. Deze niercellen zijn ontwikkeld door in primaire niercellen twee genen in te brengen middels moleculair-biologische technieken: een temperatuur-gevoelige variant van het Simian virus 40 large-t-antigeen (SV40T), U19tsA58, en het katalytische onderdeel van humane telomerase (hTERT). Dit heeft ertoe geleid dat de cellen onsterfelijk zijn en tot grote aantallen opgekweekt kunnen worden voor gebruik in regeneratieve niertherapieën. Bovendien heeft uitvoerig onderzoek al laten zien dat de cellen diverse eigenschappen bezitten die van belang zijn voor hun functie, waaronder de aanwezigheid van diverse membraantransporteiwitten die de uitscheiding van afvalstoffen faciliteren. Dat maakt de keuze voor toepassing van deze niercellen in BAK ontwikkeling een logische stap.

Ondanks de veelbelovende eigenschappen van ciPTEC als celmodel, zullen veiligheidsaspecten zoals activering van het immuunsysteem, kankerverwekking en andere ongewenste effecten, nauwgezet onderzocht moeten worden. Dit proefschrift beschrijft het preklinische veiligheidsonderzoek van ciPTEC als niercelmodel voor de BAK. **Hoofdstuk 2** beschrijft een literatuuronderzoek naar het gebruik van genetisch-gemodificeerde cellen als therapie in diermodellen voor nierziekten. Door systematisch onderzoek te doen naar publicaties vermeld in de openbare databases PubMed en EMBASE, konden relevante wetenschappelijke artikelen worden verzameld. Alleen die artikelen die voldeden aan voorafgestelde criteria werden verder geanalyseerd en gebruikt om een uitspraak te kunnen doen over de veiligheid van genetisch-

gemodificeerde cellen voor niercelltherapie. Uit dit onderzoek bleek dat er geen grote risico's verbonden zijn aan het gebruik van genetisch-gemodificeerde cellen, hoewel door een grote verscheidenheid aan studiekarakteristieken, zoals het gekozen diermodel en wijze van celtherapie, en een suboptimale studieopzet bij het merendeel van de studies, een duidelijke uitspraak omtrent de veiligheid niet gedaan kan worden. Wij adviseren dan ook om verder onderzoek te blijven doen naar de effectiviteit van celtherapieën voor nierziekten, waarbij ook de veiligheid als eindpunt meegenomen wordt.

Naast de bewezen functie om afvalstoffen actief uit te kunnen scheiden zou een ciPTEC-bevattende BAK ook hormonale functies moeten kunnen vervangen. **Hoofstuk 3** besteedt aandacht aan de mogelijkheid van ciPTEC om de actieve vorm van vitamine D, het 1α,25-dihydroxy-vitamine D_3 ($1,25(OH)_2D_3$) ook wel bekend als calcitriol, te activeren vanuit de precursor 25-hydroxy-vitamine D_3 ($25(OH)D_3$), om deze vervolgens uit te scheiden. Deze functie kon worden aangetoond en de activatie en uitscheiding van calcitriol waren niet aangedaan onder uremische omstandigheden, zoals die bij nierpatiënten worden waargenomen. Van vitamine D kon verder worden aangetoond dat het de levensvatbaarheid en het metabolisme van de cel positief kan beinvloeden, alsmede het herstel van een beschadigde cellaag kan bevorderen. Al deze aspecten zijn van groot belang in een goed functionerende BAK.

In **hoofdstuk 4** staat onderzoek naar de mogelijke reactie van het immuunsysteem op ciPTEC centraal. Een aantal moleculen die het immuunsysteem kunnen activeren, zoals CD40 en HLA klasse I moleculen, bleken op het membraan van de niercellen aanwezig te zijn en door ontstekingsfactoren te worden geïnduceerd. Echter andere belangrijke moleculen, waaronder HLA klasse II moleculen en CD80 en CD86, bleken niet of nauwelijks aanwezig te zijn. Wanneer humane witte bloedcellen werden blootgesteld aan ciPTEC werd geen activatie van deze bloedcellen waargenomen. Ook niet wanneer de ciPTEC eerst extra geactiveerd werden. Bovendien, wanneer BAK-relevante omstandigheden werden nagebootst door de niercellen op polyethersulfonmembranen te kweken en ontstekingscondities na te bootsten, werd eveneens geen activatie van deze bloedcellen gevonden noch werd de barrièrefunctie van de laag van ciPTEC aangetast.

De immuunveiligheid van ciPTEC is in **hoofdstuk 5** verder onderzocht door gebruik te maken van een opgeschaald systeem, bestaande uit een bioreactor met meerdere nierbuisjes. In dit systeem werd de uitscheiding van de ontstekingsbevorderende en immuunmodulerende factoren interleukine-6, -8, tumornecrosisfactor-alfa en oplosbaar HLA klasse I onderzocht. De resultaten laten zien dat er een gepolariseerde afgifte van de factoren is, namelijk naar het apicale compartiment dat het afvalcompartiment voorstelt en niet naar het basolaterale compartiment dat in contact zal komen te staan met de bloedsomloop. Bovendien kon aan de hand van het transport van een modelsubstraat de effectiviteit van het systeem worden aangetoond. Gezamenlijk laten de resultaten van hoofdstuk 4 en 5 zien dat opschaling van een BAK-systeem met ciPTEC een veilige benadering is.

In **hoofdstuk 6** is onderzoek gedaan naar de mogelijke kankerverwekkende effecten van ciPTEC. Hiervoor is een serie aan *in vitro* experimenten verricht waarbij de eigenschappen van ciPTEC gekweekt bij 33 °C werden vergeleken met die gekweekt bij 37 °C, omdat de cellen

onsterfelijk zijn gemaakt door transductie met het temperatuurgevoelige SV40T-gen. Zodra de cellen bij 37 °C werden gekweekt nam de aanwezigheid van het SV40T-eiwit sterk af, net als de celgroei. Daarentegen namen epitheliale eigenschappen van de cellen juist toe, waaronder de gevoeligheid voor p53-gemediëerde apoptosis. Bovendien vond zonder hechting aan een dragend oppervlak of contacten tussen cellen geen celgroei plaats. Tevens werd de genetische stabiliteit en de integratie van de transgenen in het genoom onderzocht, waaruit bleek dat de endogene functie van een aantal genen niet aangedaan is door de immortalisatie. Tenslotte kon worden aangetoond dat ook in proefdieren, in immuundeficiënte ratten, over een periode van meer dan 4 maanden geen tumoren of metastasen werden gevonden na onderhuidse toediening van cellen of ciPTEC-materialen. Op basis van deze resultaten kunnen we concluderen dat ciPTEC een veilige optie is voor toekomstige BAK toepassingen.

Ten slotte geeft **hoofdstuk 7** een verdiepende discussie van de verkregen resultaten alsmede de mogelijkheden voor toekomstige toepassingen van een BAK-systeem. De in dit proefschrift bewezen veiligheid van ciPTEC als celmodel moedigt verder onderzoek aan naar de effectiviteit en veiligheid van het systeem in diermodellen als opstap naar toekomstig klinisch onderzoek.

SAŽETAK NA SRPSKOM

Korak bliže ka veštačkom bubregu: preklinička evaluacija bezbednosti

Bubrezi igraju važnu ulogu u brojnim procesima neophodnim za normalno funkcionisanje organizma i održavanje homeostaze, uključujući regulaciju izlučivanja vode i koncentraciju elektrolita, kiselinsko-baznu ravnotežu, krvni pritisak, kao i endokrine i metaboličke funkcije i eliminisanje štetnih produkata metabolizma i ksenobiotika. Stoga je razumljivo da bolesti bubrega i gubitak bubrežnih funkcija mogu prouzrokovati ozbiljne kliničke manifestacije. Trenutno dostupne opcije za lečenje pacijenata obolelih od hronične bubrežne insuficijencije podrazumevaju hemodijalizu, peritonealnu dijalizu i transplantaciju bubrega. Međutim, imajući u vidu izrazit nedostatak donora organa za transplantaciju bubrega s jedne strane, i činjenice da dijaliza ne pruža kompletno uklanjanje uremijskih metabolita, naročito jedinjenja vezanih za proteine plazme, s druge strane, postoje nekoliko različitih inovativnih opcija za tretman bubrežne insuficijencije koje su predmet aktuelnih naučnih razvića i istraživanja. One bi trebalo da poboljšaju ne samo klinički ishod već i opšte zdravstveno stanje pacijenata bubrežnih oboljenja. Jedno od najprimamljivijih i najpovoljnijih rešenja je takozvani veštački ili bioarteficijelni bubreg, uređaj koji predstavlja proizvod kombinovanja savremenih inovativnih biotehnoloških procedura i principa tkivnog inženjeringa koji se inače i koriste za razvoj naprednih medicinskih terapijskih oprema. Veštački bubreg ima za cilj da nadoknadi gubitak osnovnih funkcija bubrega, pomoću kombinovanja takozvanih kalupa (skafolda) kao što su polimerska šuplja membranska vlakna i proksimalne tubularne epitelne ćelije, stvarajući tako biološki funkcionalisane bubrežne cevčice koje bi mogle zameniti ne samo ekskretorne, već i endokrine, metaboličke i regulatorne funkcije bubrega. Ova svojstva su trenutno odsutna u standardnoj dijalizi.

Što se tiče ćelija, jedna od veoma privlačnih opcija za primenu u veštačkom bubregu su takozvane uslovno imortalizovane humane proksimalne tubularne epitelne ćelije (ciPTEC). Ove ćelije su kreirane zahvaljujući imortalizaciji primarnih proksimalnih tubularnih ćelija posredstvom dvostruke retroviralne transdukcije: mutirana forma SV40 velikog T antigena (SV40T) koja je osetljiva na temperaturu, U19tsA58, i katalitička podjedinica humane telomeraze (hTERT). Ovakva vrsta imortalizacije omogućava neprekidnu ekspanziju i dostupnost ćelija, znatno olakšavajući prevazilaženje problema vezanih za nedostatak izvora ćelija, što je često prisutan problem u slučaju primene primarnih ćelija u svrhe tkivnog inženjeringa. Osim toga, pošto su ove ćelije snabdevene nekim od najvažnijih transportnih proteina (transportera), uključujući između ostalih, organske anjonske transportere 1 i 3 (OAT1/3), organski katjonski transporter 2 (OCT2), transportni protein otpornosti raka dojke (BCRP), P-glikoprotein (P-gp), transportere za ekstruziju višestrukih lekova i toksina 1 i 2 (MATE1/2) i transportere odgovorne za otpornost na višestruke lekove 2 i 4 (MRP2/4), od kojih su svi sposobni da aktivno uklanjaju mnoge štetne proizvode metabolizma, nije iznenađujuće što upravo ove ćelije (ciPTEC) predstavljaju najočigledniji izbor za primenu u veštačkom bubregu.

Uprkos ovim korisnim svojstvima ciPTEC ćelija za upotrebu u veštačkom bubregu, pojedini problemi koji se tiču bezbednosti korišćenja genetski modifikovanih ćelija u medicinske svrhe, kao što su aktivacija imunog sistema, tumorigeni potencijal i drugi neželjeni efekti, moraju biti pažljivo ispitani.

Ova disertacija se bavi ispitivanjem bezbednosti ciPTEC ćelija za upotrebu u veštačkom bubregu. **Drugo poglavlje** pruža opšti pregled trenutno dostupne literature koja se tiče upotrebe genetski modifikovanih ćelija u životinjskim modelima bubrežnih bolesti u svrhe ćelijske terapije. Relevantne naučne publikacije su prikupljene pomoću sistematske pretrage literature u PubMed i EMBASE bazama podataka. Naučni članci koji su zadovoljili prethodno definisane kriterijume uključivanja bili su dalje analizirani i iskorišćeni za određivanje bezbednosti genetski modifikovanih ćelija. Dobijeni rezultati su pokazali da, generalno, ćelijska terapija za bubrežne bolesti ne predstavlja izrazit rizik, bar što se tiče tumorigenog i teratogenog dejstva. Međutim, usled velike raznovrsnosti u karakteristikama studija (životinjske vrste, model bubrežne bolesti, vrsta ćelijske terapije) i činjenice da većina istih ima suboptimalan ili neprikladan dizajn studije, bilo je prilično izazovno dobiti opšti pregled o bezbednosnim aspektima ćelijske terapije. Stoga su dalja istraživanja i studije sa adekvatnim dizajnom za ispitivanje bezbednosti ćelijske terapije za bubrežna oboljenja od krucijalnog značaja i više nego neophodna.

Pored već dokazane aktivnosti transporta uremijskih jedinjenja, veštački bubreg koji sadrži ciPTEC ćelije može imati dodatnu prednost u vidu endokrinih funkcija koje su u obolelim bubrezima izgubljene i ne mogu biti nadoknađene dijalizom. Glavni cilj **trećeg poglavlja** bio je ispitivanje sposobnosti ciPTEC ćelija da aktiviraju i luče biološki najaktivniju formu vitamina D, 1α,25-dihidroksivitamin D_3 ($1,25(OH)_2D_3$), takođe poznat i kao kalcitriol, počevši od prekursora 25-hidroksivitamina D_3 ($25(OH)D_3$). Najvažniji nalazi ovog poglavlja su ta da su ciPTEC ćelije sposobne da aktiviraju vitamin D u hemijskoj reakciji katalizovanoj od strane enzima 1α-hidroksilaza i da ta reakcija nije kompromitovana prisustvom uremijskih jedinjenja, uslovi tipični za pacijente bubrežnih bolesti. Osim toga, rezultati su pokazali da vitamin D poseduje i blagotvorno dejstvo na ciPTEC ćelije u uremičnim uslovima, naročito na vitalnost ćelija, poboljšanje inflamatornog i oksidativnog statusa ćelija, kao i obnavljanje i jačanje funkcije transepitelne barijere. Svi ovi upravo navedeni aspekti mogu biti od ključnog značaja za pravilno funkcionisanje veštačkog bubrega.

Četvrto poglavlje se bavi alostimulatornom sposobnošću ciPTEC ćelija i ispitivanjem bezbednosnih pitanja ćelijske terapije odnosno na ćelijsku imunogenost. CiPTEC ćelije izolovane iz bubrežnog tkiva (ciPTEC-T1) i urina (ciPTEC-U) bile su podvrgnute, pod različitim stimulatornim uslovima, veoma temeljnoj karakterizaciji ekspresije i sekrecije najvažnijih molekula neophodnih za aktivaciju aloimunog odgovora. Iako se pokazalo da obe ćelijske linije izražavaju CD40 i HLA (Ljudski leukocitni antigen) molekule iz klase I, čija se ekspresija znatno povećava nakon izlaganja interferonu-gama (IFN-γ) i bakterijskom lipopolisaharidu (LPS), ekspresija HLA molekula iz klase II i kostimulatornih molekula CD80 i CD86 je bila veoma niska ili potpuno odsutna. Takođe, ciPTEC ćelije ne poseduju direktan alostimulatorni efekat s obzirom na to da se pokazalo da nisu bile u stanju da indukuju aktivaciju i proliferaciju mononuklearnih ćelija periferne krvi (PBMC) *in vitro*. Najzad, u uslovima koji su relevantni za veštački bubreg, u kojima

su ciPTEC i PBMC ćelije odvojene biološki funkcionalisanom polietersulfonskom membranom, pokazalo se da čak ni jaki inflamatorni (LPS) ili uremični (plazma pacijenata obolelih od hronične bubrežne insuficijencije) uslovi nisu ugrozili transepitelnu barijeru ciPTEC ćelija, što je potvrđeno merenjem difuzije FITC-inulina.

Imunogena svojstva ciPTEC ćelija studirana u četvrtom poglavlju bila su dalje produbljena u **petom poglavlju**. Naime, u ovom poglavlju je ispitana sekrecija proinflamatornih i imunomodulatorskih posrednika uključujući interleukin-6 (IL-6), interleukin-8 (IL-8), faktor nekroze tumora-alfa (TNF-α) i solubilne HLA molekule klase I, unutar prvog prototipa veštačkog bubrega koji sadrži višestruka polimerska šuplja vlakna sa ciPTEC ćelijama. Dobijeni rezultati su pokazali da postoji potpuno polarizovana sekrecija spomenutih molekularnih medijatora, pretežno orijentisana ka apikalnoj strani, tj. odeljku koji bi sadržao dijalizat, u odnosu na bazolateralnu stranu koja odgovara odeljku koji bi sadržao telesnu tečnost pacijenta. Integritet transepitelne barijere ciPTEC ćelija i transepitelni transport indoksil sulfata su takođe potvrđeni u predstavljenom modelu. Zajedno, ovi rezultati ukazuju na uspešnu izgradnju prvog prototipa veštačkog bubrega koji sadrži višestruka polimerska šuplja vlakna sa funkcionalnim jednoslojem epitelnih ćelija i pokazuju polarizovanu sekreciju inflamatornih molekula za bezbedniju buduću primenu.

Potencijalni tumorigeni i onkogeni efekat ciPTEC ćelija je bio predmet ispitivanja **šestog poglavlja**. Čitav niz *in vitro* eksperimenata je korišćen za studiranje i upoređivanje ponašanja ciPTEC ćelija na permisivnoj (33 °C) i nepermisivnoj (37 °C) temperaturi, uzevši u obzir prirodu ekspresije i funkcije SV40T mutanta upotrebljenog za imortalizaciju ćelija. Rezultati pokazuju da je ekspresija SV40T antigena značajno smanjena na 37 °C, čak i nakon samo jednog dana inkubacije. Što se tiče brzine razmnožavanja i rasprostranjenosti ćelija po fazama ćelijskog ciklusa, dobijeni rezultati ukazuju na to da se ćelije ponašaju shodno zavisnosti ekspresije SV40T antigena od temperature, sa povećanom proliferacijom na permisivnoj temperaturi. Osim toga, pokazalo se i da su ćelije koje rastu na nepermisivnoj temperaturi senzitivne na apoptozu posredovanu p53 proteinom. Uz to, rast ćelija nije opažen u odsustvu čvrstog oslonca (matriksa), a takođe pokazalo se i da ćelije podležu fenomenu kontaktne inhibicije. Genetička stabilnost ciPTEC ćelija je utvrđena pomoću analize kariotipa i integracije transgena unutar ćelijskog genoma, što je pokazalo da transdukcija transgena nema štetnih posledica po ekspresiju i funkciju endogenih gena (npr. sposobnost endocitoze). Uprkos prisustvu retkih događaja ćelijske invazije kroz vanćelijski matriks i nestabilnom kariotipu usled dugotrajne ćelijske kulture, *in vivo* eksperimenti su pokazali odsustvo tumorigenog i onkogenog efekta ciPTEC ćelija nakon njihovog ubrizgavanja u imunološki kompromitovane, takozvane gole, atimične pacove. Na osnovu dobijenih rezultata u ovom poglavlju, ciPTEC ćelije se mogu smatrati prilično bezbednom opcijom za buduće aplikacije veštačkog bubrega.

Najzad, **sedmo poglavlje** pruža iscrpnu diskusiju rezultata kao i predloge i perspektive za dalji razvoj veštačkog bubrega. Rezultati o bezbednosti ciPTEC ćelija predstavljeni u ovoj disertaciji, uključujući odsustvo imunogenog i tumorigenog efekta i prisustvo željenih endokrinih funkcija,

pružaju značajan doprinos razvoju veštačkog bubrega baziranog na ciPTEC ćelijama, i ohrabruju dalja preklinička testiranja u životinjama sa bubrežnom insuficijencijom, što je preduslov za buduća klinička ispitivanja.

LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	1α,25-dihydroxy-vitamin D_3
$25(OH)D_3$	25-hydroxy-vitamin D_3
2D	Two-dimensional
3D	Three-dimensional

A

ABC	ATP-binding cassette
ADAM17	A disintegrin and metalloproteinase domain 17
ADMSC	Adipose-derived mesenchymal stem cell
afMSC	Amniotic fluid-derived mesenchymal stem cell
AFU	Arbitrary fluorescence unit
AKI	Acute kidney injury
ANOVA	Analysis of variance
APC	Antigen presenting cells
AQP1	Aquaporin 1
ATMPs	Advanced therapy medicinal products
ATP	Adenosine triphosphate

B

BAK	Bioartificial kidney
Bax	B-cell lymphoma 2 associated X protein
Bcl2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BCRP	Breast cancer resistance protein
BLI	Bioluminescence imaging
BMC	Bone marrow cell
BM-MSC	Bone marrow-derived mesenchymal stem cell
BMP-7	Bone morphogenetic protein 7
BRECS	Bioartificial Renal Epithelial Cell System
BSA	Bovine serum albumin
BTD	Bioartificial tubule device
Bub-1	Budding uninhibited by benzimidazoles 1
BVDV	Bovine viral diarrhea virus

C

Ca_2^+	Calcium
CAT	Committee for Advanced Therapies
CCD	Centrale commissie dierproeven
CD40	Cluster of Differentiation 40

CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
cDNA	complementary deoxyribonucleic acid
CFDA-SE; CFSE	Carboxyfluorescein diacetate succinimidyl ester
ciPTEC	Conditionally immortalized proximal tubule epithelial cell
ciPTEC-OAT1	Conditionally immortalized proximal tubule epithelial cells, overexpressing OAT1
ciPTEC-T1	conditionally immortalized proximal tubule epithelial cell, kidney tissue-derived
ciPTEC-U	Conditionally immortalized proximal tubule epithelial cell, urine-derived
CK-18	Cytokeratin 18
CKD	Chronic kidney disease
Cl^-	Chloride
CM	Conditioned medium
CM	Complete medium
CO_2	Carbon dioxide
ConA	Concanavalin A
CPF	Colonies per field
CRF	Chronic renal failure
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9
Ct	Threshold cycle
CWF	Clean water flux
CXCR4	C-X-C chemokine receptor type 4
CYP	Cytochrome P450

D

Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DHS	DNase I hypersensitive site
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E

EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome antigen 1
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
EMA	European Medicine Agency
EPC	Endothelial progenitor cell

EPO	Erythropoietin
ESC	Embryonic stem cell
ESRD	End-stage renal disease
EYFP	Enhanced yellow fluorescent protein

F

FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate

G

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCP	Good Clinical Practice
GFP	Green fluorescent protein
GFR	Glomerular filtration rate
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GVP	Good Pharmacovigilance Practice

H

H_2DCFDA	2',7'-dichlorofluorescein diacetate
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HCO_3^-	Bicarbonate
HeLa	Henrietta Lacks cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFM	Hollow fiber membrane
HGF	Hepatocyte growth factor
HK-2	Human kidney-2 cells
HLA	Human Leukocyte Antigen
hPAP	Human placental alkaline phosphatase
HPLC	High-performance liquid chromatography
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase 1
HRP	Horseradish peroxidase
hRPTEC	Human renal proximal tubular epithelial cell
HSC	Hematopoietic stem cell
HSPC	Hematopietic stem and progenitor cell
hTERT	Human telomerase reverse transcriptase
hucMSC	Human-umbilical cord-derived mesenchymal stem cell

I

I/R	Ischemia-reperfusion
IAA	Indole-3-acetic acid
IBD	Inflammatory bowel diseases
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
iPSCs	induced pluripotent stem cells
IS	Indoxyl sulfate
ISH	*In situ* hybridization
IVD	Instantie voor dierenwelzijn
IVF	*In vitro* fertilization

K

K^+	Potassium
KA	Kynurenic acid
KCl	Potassium chloride
kDa	Kilodalton
KH	Krebs-Henseleit
$KHCO_3$	Potassium bicarbonate
KHH	Krebs-Henseleit HEPES
Klf4	Kruppel-like factor 4

L

L	Membrane permeance
L-DOPA	L-3,4-dihydroxyphenylalanine
LLC-PK_1	Lilly Laboratories Cell Porcine Kidney cells
LPS	Lipopolysaccharide
LRP2	Low density lipoprotein-related protein 2
LTRs	Long-terminal repeats

M

MATE1	Multidrug and toxin extrusion protein 1

MATE2	Multidrug and toxin extrusion protein 2
MCP-1	Monocyte chemoattractant protein 1
MDCK	Madin-Darby Canine Kidney cells
Mdm2	Mouse double minute 2 homolog
MFI	Median fluorescent intensity
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MMP 2	Matrix metalloproteinase 2
MMP 9	Matrix metalloproteinase 9
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRP2	Multidrug resistance protein 2
MRP4	Multidrug resistance protein 4
MRPC	Mouse renal progenitor cell
MSC	Mesenchymal stem cell
Mw	Molecular weight

N

Na^+	Sodium
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
NMD	Nonsense-mediated decay
NMR	Nuclear magnetic resonance

O

OAT1	Organic anion transporter 1
OAT3	Organic anion transporter 3
OAT4	Organic anion transporter 4
OATP4C1	Organic anion transporting peptide 4C1
OCT1	Organic cation transporters 1
OCT2	Organic cation transporters 2
OCT3	Organic cation transporters 3
Oct-4	Octamer-binding transcription factor 4
OCTN1	Organic cation/carnitine transporter 1
OCTN2	Organic cation/carnitine transporter 2

P

$p16^{INK4a}$	Cyclin-dependent kinase inhibitor 4A
$p21^{Cip1}$	Cyclin-dependent kinase-interacting protein 1
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
pCG	p-cresyl glucuronide
PCR	Polymerase chain reaction
PCR-SSO	Sequence-specific oligonucleotide polymerase chain reaction
pCS	p-cresyl sulfate
PDGF	Platelet-derived growth factor
PERT	Product-enhanced reverse transcriptase
PES	Polyethersulfone
PFA	Paraformaldehyde
P-gp	P-glycoprotein
PHA-P	Phytohemagglutinin-P
PI	Propidium iodide
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PKD	Polycystic kidney disease
pRb	Retinoblastoma protein
PTEC	Proximal tubule epithelial cell
PVDF	Polyvinylidene difluoride
PVP	Polyvinylpyrrolidone

R

RCL	Replication-competent lentiviruses
RCR	Replication-competent retroviruses
RFP	Red fluorescent protein
RIA	Radioimmunoassay
RIPA	radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT	Room temperature

S

SAA1	Serum amyloid A1
SCID	Severe combined immunodeficient
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SEM	Scanning electron microscope
SFM	Serum-free medium
sHLA	Soluble HLA
SINEs	Short interspersed elements
siRNA	Small interfering ribonucleic acid

SLC	Solute carrier
SMA	Smooth muscle actin
SNP	Single nucleotide polymorphism
Sox2	Sex determining region Y-box 2
SPIO	Superparamagnetic iron oxide
SRC	Selected renal cells
SRY	Sex-determining region Y
STRs	Short tandem repeats
SV40T	Simian virus 40 Large T antigen

T

TBE	Tris-Borate-EDTA
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TI	Transcriptional interference
TLA	Targeted locus-amplification
TMP	Transmembrane pressure
TNF-α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

U

URAT1	Urate reuptake transporter 1
UT	Uremic toxin
UUO	Unilateral ureter obstruction
UV/VIS	Ultraviolet–visible spectroscopy

V

VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VEP	Variant Effect Predictor
Vero	Verda reno cells
vWF	Von Willebrand factor

W

WEBAK	Wearable bioartificial kidney
WHO	World Health Organization

Z

ZO-1	Zonula occludens-1

CHAPTER IX

CURRICULUM VITAE

LIST OF PUBLICATIONS

ACKNOWLEDGMENTS

CURRICULUM VITAE

Miloš Mihajlović was born on 11th April 1989 in Požarevac, Serbia. After graduating from high school in 2008, he moved to Trieste, Italy where he studied at the University of Trieste, Faculty of Medicine and Surgery. In July 2011, he obtained his B.Sc. degree in Biotechnology, and in October 2013 he graduated *cum laude* with a M.Sc. degree in Medical Biotechnology (study program Molecular Medicine). He carried out his graduation project at the Italian Liver Foundation (Centro Studi Fegato - Fondazione Italiana Fegato) in AREA Science Park, Basovizza (Trieste), under the supervision of Prof. Claudio Tiribelli and Dr. Natalia Rosso, working on the development of an *in vitro* model of alcoholic liver disease. After the graduation, in December 2013, he started his PhD research at the Department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences in Nijmegen, The Netherlands. He was enrolled as an early stage researcher (ESR) within the BIOART Initial Training Network (EU-FP7, Marie Skłodowska-Curie actions), under the supervision of Prof. Dr. R. Masereeuw and Prof. Dr. L.B.Hilbrands. His research project was focused on the preclinical safety evaluation of bioartificial kidney for the improvement of kidney disease treatment. In July 2015, he moved to the Utrecht Institute for Pharmaceutical Sciences at Utrecht University, following the appointment of Prof. Dr. R. Masereeuw as Professor of Experimental Pharmacology. Here, he was enrolled in Drug Innovation PhD program and continued working on his doctoral research project, completing it in February 2018. The most important results from his research are reported in this dissertation. As of May 2018, he is appointed as a postdoctoral researcher in the same research group at Utrecht University, where he continues to study the functional and safety aspects of bioartificial kidney in animal models of chronic kidney disease (in collaboration with Baxter, Deutschland).

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ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest and sincere gratitude to **Roos** for so many things she has done during the past 4 years in order to make it possible for me to be at this point of my research career. Thank you for giving me the opportunity to be a part of your group and learn so much on this exciting topic. Thank you for all the support, guidance and for sharing your knowledge and experience. Thank you for believing in me and in my project even when I didn't. Thank you for the tremendous amount of time and effort you invested in my professional growth and to make me feel at ease. Thank you for being a caring and considerate promotor and supervisor. Thank you for everything! I would also like to thank my second promotor and supervisor, **Luuk**, for being always present, even after the distance increased by more than 80 km. Thank you for giving, sharing, suggesting, even questioning. Thank you for helping me improve my skills and way of thinking. Thank you for your professional (medical) insight into this important, and until then, to me not very familiar world of kidney patients and their everyday struggle. Thank you for all your guidance and support during the past few years. I really appreciate everything you have done. It has been invaluable having you on board during this journey. Thank you!

Next, I also want to thank **Bert** and **Joost** for being part of the team and for contributing to this project with helpful insights and suggestions. I am very grateful for your support over these 4 years and it was a real pleasure being able to share with you all my progresses, no matter how small or big they were. **Martijn**, thank you so much for all discussions and contributions to the project. Your participation in the BioKid meetings was always very helpful, as much as your inexhaustible experience and knowledge of ciPTECs.

I want to thank **Dimitrios** for all his efforts in making possible the Bioart project, not only for me but for all other people involved, and for coordinating it in the best possible way. Also, thank you for being always closely involved in my project and available to discuss its progress, whether it was in Nijmegen, Utrecht, Twente, via Skype calls or in hotel rooms during Bioart meetings.

Furthermore, my sincere gratitude goes to the **members of the Committee**, Prof. Dr. Stéphane Burtey, Prof. Dr. Irma Joosten, Dr. Frank Redegeld, Prof. Dr. Niels Geijsen and Prof. Dr. Ton Rabelink for accepting to be part of the thesis Committee and for reading critically the manuscript.

Next, I would like to express my immense gratitude to **Rob** and Kim from SYRCLE for all the help and support in preparing and writing my systematic review. **Kim**, I really have to say thank you for bearing with me and being very patient during this extremely long process.

I would also like to thank DMPC pathologists, **Alain** and **Laura,** for their great help with histopathological analyses.

Another sky high thank you goes to my paranymphs. **Jitske**, it has been great having you around since the very beginning and even now standing right next to me in this very moment. I am grateful for all your help. Many things would have been very difficult if it hadn't been for you – the pillar of the lab. **Michele**, non ci sono parole per ringraziarti per tutto quello che abbiamo

condiviso in questi 4 anni, dal primo giorno in cui, completamente spaesati (almeno io), abbiamo incominciato questa "dura" esperienza fino a questo momento. Grazie per tutte le avventure e gli imprevisti di ogni giorno, che adesso mi e' impossibile ricordarli tutti. Grazie di cuore!

Next, I would like to thank all colleagues and friends who have helped me in any way in this process. Here, I must start with **Manoe**. Thank you for always trying to find (and finding) a way, a solution, an explanation, a fix, an 'anything'. Your positive attitude towards science and life and your calmness are really inspiring. Thank you for your contribution to my project. And also, thank you for often sharing Utrecht-Nijmegen NS experiences (the good and the not so good ones). A special thank you goes to **Silvia**, for helpful discussions and for being the most energizing colleague with so many great ideas. The two "old/new – Nijmegen/Utrecht – student/PhD" faces are also appreciated for sharing enjoyable, every day lab experiences: Katja and Amer. **Katja**, I admire very much your challenge-accepting trait and surprisingly special sense of humor, and **Amer** (I really don't know what to say but), among all the things, I particularly appreciate your constant giggling, "dribbling" nature and your special efforts in trying to embarrass me (and yourself) in public with fake, tuberculosis-like sudden cough attacks ☺. Joking aside, you are both great colleagues and on the right way of becoming excellent researchers. My new neighbor, **Anne Metje**, I really appreciate your thorough and systematic way of working – you did a great progress from being twice an excellent student in the Pharmacology group, via Boston and to the PhD in Pharmacology again. Also, I am really thankful for reminding me constantly when it was the right time for cookies. **Pedro**, thank you for teaching me how to use FACS when I first started in Nijmegen, and for letting me teach you how to use it in Utrecht few years later ☺. Your seemingly casual and laid-back approach to the difficulties of a PhD and ability to start new, successful career path is both comforting and promising. Thank you for always being willing to help. **Haysam**, thank you for all the nice moments we shared as a team in the Bioart meetings, and I am really looking forward to your future contributions to the Pharmacology group. **Koen**, thank you for your immense help with the animal experiments.

I really cannot proceed without thanking to the laughing trio from the corner, behind my back, that specialized in breaking the daily office routine with sudden LOL and silly jokes: **Paul**, for all discussions about trustability, gambling games, magician tricks, and for sharpening my skepticism ☺ and I wish you success for the rest of your PhD journey. **Ling**, for always smiling, laughing and making jokes (even at your own expense). Wish you a lot of success as you are now 'the next in line'. **Paula**, for always being extremely honest and transparent and for controlling the other two when they were riling up each other ☺.

A special thank you goes to **Natalia** (Sanchez Romero) for being temporarily a part of Pharmacology group and bringing optimistic and cheerful spirit, other than helping with so many practical lab things and participating in discussions. Also to **Pedro B**, for inspiring talks and nice moments at ESAO meetings.

Yingxin, for being genuine and honest person and for not getting mad at me when I was teasing you. Sometimes it's really hard to resist knowing your funny reactions ☺.

Gemma, thank you for your selfless support and assistance with FACS, for your help around the lab and for managing orders and other financial issues so efficiently.

I would like to express a very special gratitude to the secretary office, Lidija, **Marga** and **Brenda**, who were efficiently dealing with all bureaucracy issues at any time**. Lidija**, hvala puno za odgovore na sva moja pitanja i rešenja za mnoge administrativne problemčiće, a naročito za viceve i šale.

Next, I would like to thank all students who at some point during the past few years worked with me on this project. **Thom**, for taking initiative and contributing to the first phases of the unexpectedly long journey of writing the systematic review. **Annemarie**, for your dedication, ambition and a big desire to perform a successful internship. **Sonja**, for your organizational skills, taking initiative, determination and right approach to the research. **Miriam**, for your enormous help, willingness and courage to be involved in the animal study until the end. Thank you for taking such a good care of Ursula. **Daria**, for being very energetic, willing to learn and enthusiastic. **Sam** (my 'overrated' student), for your unusual, eccentric and outside of the box way of thinking, critical touch with the right amount of perfectionism in all tasks you undertook. And of course for reminding me of myself at times ☺. Thank you all for your hard work and I truly hope that you enjoyed, more or less, and above all learned something that can help you in your future careers.

I also want to thank all current and former colleagues from Pharmacology department for welcoming me in Utrecht, making me feel comfortable and maintaining that feeling until this day and who have made this experience much more enjoyable: **Johan**, **Aletta**, **Paul H**, **Frank, Mara**, **Marlotte**, **Atanaska**, **Suzanne**, **Veronica**, **Suzan**, **Melanie**, **Kirsten**, **Carla**, **Michele B**, **Susan**, **Jing**, **Yulong**, **Aurora**, **Negisa**, **Hamed**, **Adel**, **Sandra**, **Charlotte**, **Soheil**, **Selda**, **Mitch**, **Mojtaba**, **Bart**, **Monika**, **Astrid**, **Linette**, **Erik**, **Gert**, all former students and others whom, unintentionally, I have forgotten to mention.

Next, I would like to thank a few people from University of Twente. **Natalia**, the most eccentric persona within the Bioart project, irreplaceable and key factor of the WP2, and above everything the most interesting and funny collaborator I have ever worked with. Thank you for all nice moments during the Bioart meetings, for making a big deal out of my visits to Twente every time, for teaching me and trying to pass on the knowledge of modules preparation and for our successful collaborations. It was a great pleasure working with you. Also, thank you for sharing food at every possible occasion, whether it was stroopwafels, carrots or simply bread. **Denys**, another fellow Bioartist, and **Ilaria G**, l'altro biotecnologo triestino 'importato' in Olanda, thank you for your warm welcome and nice talks every time I came to visit Twente. Finally, thank you Dimitrios (again) and **Karin** for taking such a good care of the Bioart and Bioartists at all occasions during the past years.

I want to say a big thank you to all **Bioartist PIs** and fellow PhD and postdoc colleagues: **Vittoria**, **Valentina**, **Ilona**, **Antonietta**, **Shervin**, **Danial**, **Dmytro**, **Marcos**, **Aniela**, **Katarzyna** (and again Michele, Natalia, Denys and Haysam). It was an extraordinal experience and fun meeting you around Europe every few months and sharing with you some of the most memorable events of my PhD journey. I am really grateful for getting the chance to know you all.

Finally, I cannot finish this section of the thesis without mentioning all good people from the Department of Pharmacology and Toxicology in Nijmegen who were the first ones to welcome me, not only in the lab but also in the Netherlands. I owe you all so much! **Janny** for taking care of orders, being my mentor in the radioisotope lab and trying to teach me some Dutch. **Marieke** for sharing the lab corner and for all the tips related to successful culturing of ciPTECs. **Tom N** for being always willing to share and help and for all good talks. I wish you a lot of success in Sweden. **Maarten** and **Lindsey** for your positive attitude, funny stories and enjoyable moments. **Tom S** for making possible our Jitske-mediated collaboration and **Karl** for being good lab neighbor and for interesting talks at any time. **Jelle**, even though we spent very little time together in the lab, it was always great hanging out together and chatting. I wish you a lot of success for the end of your PhD. A big thank you to all other group members for being an important part of my 'first' PhD: **Frans**, **Jan**, **Rick**, **Jeroen**, **Ria**, **Petra**, **Jeanne**, **Wendy**, **Robert**, **Lilibeth**, **Ab**, **Vivienne**, **Stijn** and anyone else whom I accidentally didn't mention. Also, even though not the full-time members of Pharm-Tox, I want to thank Carolien and Esther. **Carolien**, thank you for actively participating in the BioKid meetings and sharing your lab experience unreservedly, like setting up the flow system. **Esther**, thank you for your huge help in the lab, especially with ELISA and PBMC.

Наравно, највеће хвала иде **мојој породици** на несебичној подршци и разумевању. Ова дисертација је резултат, не само ове четири године проведене у Холандији, већ и свега што ми је пре тога било омогућено и пружено, што ми је дало прилику да идем даље. Хвала на константној бризи, саветима и подршци. Због свега тога овај успех је и ваш. Због свега тога ова дисертација је посвећена баш вама. ХВАЛА!

Yours,