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In vitro systems to study nephropharmacology: 2D *versus* 3D models

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ABSTRACT

The conventional 2-dimensional (2D) cell culture is an invaluable tool in, amongst others, cell biology and experimental pharmacology. However, cells cultured in 2D, on the top of stiff plastic plates lose their phenotypical characteristics and fail in recreating the physiological environment found *in vivo*. This is a fundamental requirement when the goal of the study is to get a rigorous predictive response of human drug action and safety. Recent approaches in the field of renal cell biology are focused on the generation of 3D cell culture models due to the more *bona fide* features that they exhibit and the fact that they are more closely related to the observed physiological conditions, and better predict *in vivo* drug handling. In this review, we describe the currently available 3D *in vitro* models of the kidney, and some future directions for studying renal drug handling, disease modeling and kidney regeneration.
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1. Introduction

The kidneys are essential organs in the homeostatic regulation of the human body, able to handle 180 L of plasma filtrate every day to finally excrete about 1.5 L per day in the form of urine containing waste products or foreign substances. This is required to keep our blood and extracellular fluid clean and chemically balanced. The organ further produces hormones (such as renin and erythropoietin), activates Vitamin D, regulates systemic electrolyte balance, pH, and the extracellular fluid. These functions are performed by approximately 1 million units, called nephrons, which can be subdivided into five sections, made up by the glomerulus, the proximal tubule, the loop of Henle, the distal convoluted tubule and the collecting duct. The three main processes that take place in the nephrons are: filtration, reabsorption and secretion (Fig. 1).

Upon entering the nephron capillaries, arterial blood flows through the glomerulus, where filtration occurs under influence of hydrodynamic forces. In a healthy kidney, only substances with a molecular weight up to 7000 Da can freely pass the glomerular filtration barrier. For large molecules, molecular size and charge determine the rate of filtration (Mutsaers et al., 2013). The filtered fluid therefore consists mainly of water and unbound solutes. Once this fluid passes from the glomerulus into the tubular lumen, it becomes part of the body's external environment. To prevent major loss of fluid, almost all of the filtered water is reabsorbed through channels present in the tubular segments of the nephron. Together with water, the proximal tubule cells reabsorb ions such as Na^+ and Ca^{2+}. Na^+ is actively transported into the extracellular fluid by the Na^+-K^+-ATPase localized on the basolateral membrane. By means of facilitated Na^+-coupled transport the proximal tubule is able to reabsorb a wide range of substances such as PO_4^{3-}, amino acids, glucose and organic metabolites (Rosenblatt et al., 2001). Furthermore, tubular transcytosis, endocytosis and pinocytosis can mediate the reabsorption of proteins, hormones and enzymes that have passed through the glomerular filtration barrier.

When the filtrate enters the loop of Henle, urinary concentration takes place (Eisenhoffer et al., 2012). Here a countercurrent exchange facilitates water reabsorption. In the (thick) ascending limb, active reuptake of Na^+, K^+ and Cl^- causes the fluid to become hyposmotic. The distal convoluted tubule then fine-tunes the electrolyte content by facilitating further sodium chloride reabsorption, potassium secretion, and adjusts Ca^{2+} and Mg^{2+}

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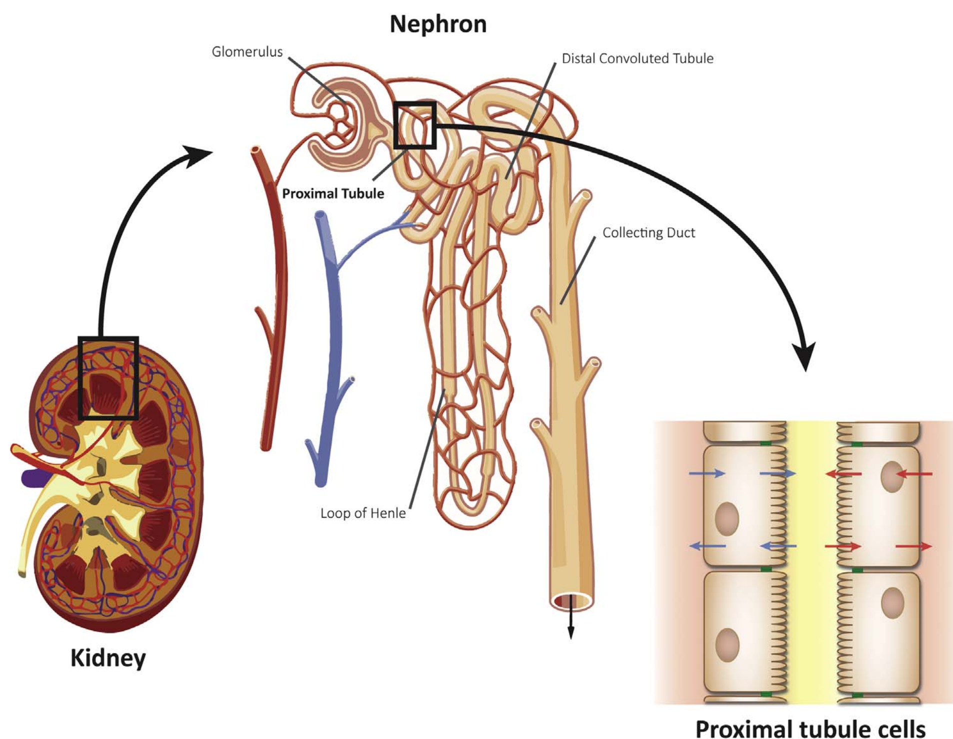

Fig. 1. Kidney and nephron morphology. The human kidney consists of approximately 1 million nephrons and each nephron has a cortical and a medullary portion. The nephron can be subdivided in five sections, made up by the glomerulus, the proximal tubule, the loop of Henle, the distal convoluted tubule and the colleting duct. This review focuses especially on the active solute transport taking place in proximal tubule epithelial cells, as proximal tubular secretion processes play an essential role in the removal of xenobiotics such as environmental chemicals, drugs, or endogenous waste products originating from metabolism.

balance (Blum, 2015). Subsequently, the collecting system, consisting of the connecting tubule and the collecting duct, make the final adjustments in urinary concentration (Eisenhoffer et al., 2012) and it decides the final excretion of potassium and protons, and sodium to some extent.

To enhance urinary excretion of substances, the kidney is able to secrete specific molecules. Secretion is very important for the maintenance of body homeostasis, acid-base balance and the removal of xenobiotics or endogenous solutes. Proximal tubular secretion processes play an essential role in the removal of xenobiotics such as environmental chemicals, drugs, or endogenous waste products originating from metabolism. Due to their high metabolic rates and exposure to toxic agents the proximal tubule cells (PTC) are more exposed to hypoxia and chemical insults than other nephron segments. Accordingly, most *in vitro* models of renal function have focused on reproducing PTC function, which is also the main cell type discussed in this review.

1.1. The proximal tubule cell in renal drug handling

The PTC are well furnished with a variety of transporters with overlapping substrate specificities that cooperate in uptake from the blood (basolateral) compartment and secretion into the pre-urine (luminal compartment). These transporters are often involved in clinically significant interactions, which may lead to unexpected changes in the plasma levels of the compounds involved and/or nephrotoxicity. PTC uptake of organic anions is mediated by members of the solute carrier (*SLC*) family known as organic anion transporter 1 and 3 (OAT1/3; *SLC22A6* and *-A8*) and the bidirectional organic anion transporting peptide 4C1 (OATP4C1; *SLCO4C1*) (Kleinman et al., 1987; Pienta et al., 1991). As the uptake of negatively charged anions is an energy consuming process, the influx transport of OAT1 and 3 is driven by their exchange for intracellular anions, such as dicarboxylates (Chen et al., 2014). The Na^+-dicarboxylate cotransporter (NaDC3; *SLC13A3*), identified in human kidney tissue in 1996, is essential for the maintenance of a cellular dicarboxylate gradient (Handler et al., 1989). The driving force for OATP4C1 has as of yet not been identified. Cellular efflux of organic anions is facilitated by members of the ATP-binding cassette (ABC) transporter family, known as the multidrug resistance proteins 2 and 4 (MRP2/4; *ABCC2* and *-C4*), and breast cancer resistance protein (BCRP; *ABCG2*), through ATP dependent transport (Terryn et al., 2007; Volpe, 2010). Furthermore, the organic anion transporter 4 (OAT4; *SLC22A11*) and the urate reuptake transporter (URAT1; *SLC22A12*) mediate the transport of organic anions by their exchange for urate (Fey-Lamprecht et al., 1998, 2000). Fig. 2 depicts a schematic model of the major organic anion as well as cation transporters in human renal proximal tubular cells.

The uptake of organic cations is mediated by the *SLC22* family of organic cation transporters (OCTs) present at the basolateral membrane of the PTC. At the brush border membrane, the *SLC47* multidrug and toxin extrusion proteins (MATEs) are expressed. OCTs and MATEs transport a wide variety of structurally unrelated organic cations (Lee et al., 2007; Ni et al., 2011; Sato et al., 2005). In the human kidney, OCT2 (*SLC22A2*) is considered one of the most important organic cation influx proteins. Though OCT1 (*SLC22A1*) and OCT3 (*SLC22A3*) are present as well, their renal expression levels are low. In contrast, their transport function in other tissues, such as liver, heart, skeletal muscle, small intestine and lung, is well described (Lee et al., 2009; Oo et al., 2011). In the kidney, the OCT2-mediated basolateral transport of organic cations occurs through facilitated electrogenic diffusion. OCT2 transport proteins make use of the internal negative membrane potential to allow organic cations to enter into the cell. For proper substrate influx, intracellular concentrations need to remain low, as transport direction is determined by the concentration gradient of the substrate. In order to retain those low intracellular levels of cationic

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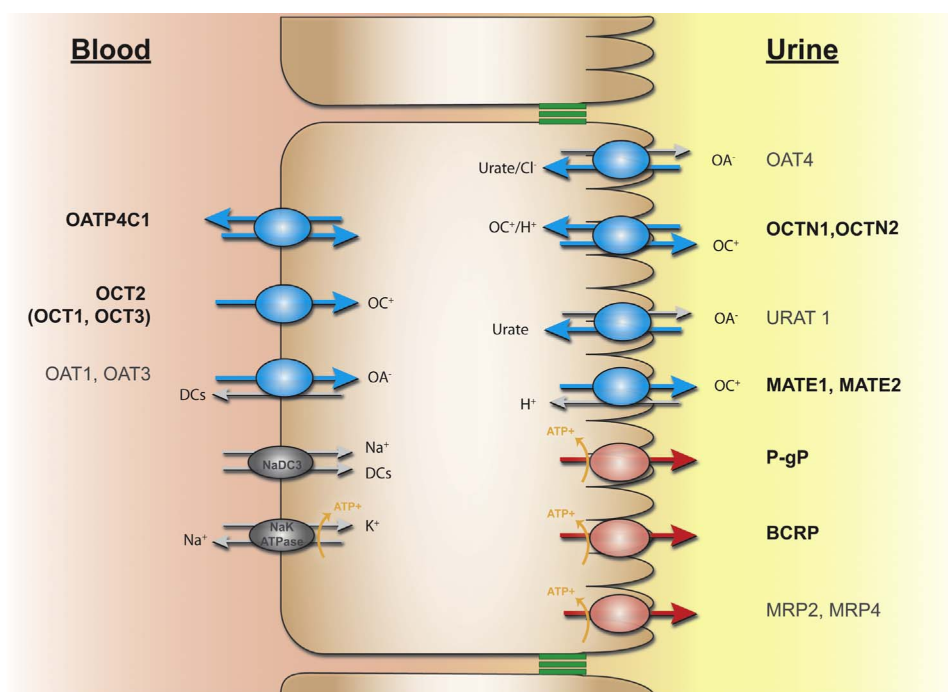

Fig. 2. Schematic model of the major organic anion (OA−)/organic cation (OC+) transporters in human renal proximal tubular cells. SLC transporters are depicted in blue and ABC transporters in red. Grey arrows depict the movement of driving ions. Transporters that are currently considered important for the clearance of organic cations are labeled In bold. More details are given in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

substrates, apical secretion follows rapidly. The apical transporters MATE1 (*SLC47A1*), MATE2 (*SLC47A2*), OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*), work in concert to mediate cation secretion on the apical brush border membrane (Chen et al., 2013; Jang et al., 2013). Apical cation secretion through these transporters is mostly driven by an H^+/organic cation antiport process (Ferrell et al., 2012). ABC transporters permeability (P)-glycoprotein (*ABCB1*; MDR1/P-gp) and the BCRP (*ABCG2*) are also involved in the apical transport of some uncharged and cationic substrates (Sirich et al., 2013; Sung and Shuler, 2012; Tehranirokh et al., 2013) (Fig. 2).

For a comprehensive overview of substrate specificities of the various transporters and their clinical implications, the reader is referred to detailed reviews, amongst others: (Koepsell, 2013; Masereeuw and Russel, 2010; Nigam et al., 2015; Wang and Sweet, 2013).

1.2. The evolution of nephropharmacology

The correct maintenance of the kidneys is essential but unfortunately, there is a high incidence of acute and chronic kidney disease (CKD), a rising global health problem with significant morbidity and mortality. These conditions affect 5–7% of the world population (Jha et al., 2013; Leung et al., 2013). In 2012 in the United States, total medical care expenditures for chronic kidney disease were near $58 billion (U.S. Renal Data System U, 2013). This problem emphasizes the need to explore new strategies to slow down or reverse renal disease progression.

Nephropharmacology is the discipline that studies the connection between clinical pharmacology and nephrology (Atkinson and Huang, 2009). This discipline started almost 50 years ago, with the contribution of Kunin et al. in 1959 when they demonstrated the dependence of drug elimination half-life ($t_{1/2}$) on renal function. Currently, the scope in nephropharmacology focuses on research related to specific drug therapy of renal diseases, as well as renal drug safety, as drug-induced nephrotoxicity contributes significantly to acute kidney failure (Loghman-Adham et al., 2012).

The development of novel drugs is both a time-consuming and cost-intensive process, and about one third fails due to toxicological concerns and/or lack of suitable testing methods capable of predicting clinical efficacy and drug safety during pre-clinical development (Kola and Landis, 2004; Stevens and Baker, 2009). For these reasons, suitable model systems for reliable pre-clinical testing are essential. Presently, the test systems in use only detect certain aspects of nephrotoxic side effects (Fliedl et al., 2014) along with the drugs efficacy. The conventional models available to study nephropharmacology include 2D PTC cultures and animal models. The problem with these models is that they do not reliably recapitulate the *in vivo* human response to drugs, and about 7% of drug candidates fail as a consequence of hidden/absence of nephrotoxicity in pre-clinical testing with 2D cell cultures or with animal models (Fuchs and Hewitt, 2011). At the same time, it is estimated that in 30–50% of all cases of severe renal failure in patients drug–induced nephrotoxicity is the cause (Fuchs and Hewitt, 2011; Pannu and Nadim, 2008). These facts highlight that the conventional methods used in nephropharmacology do not satisfactory predict the human response. Drug–induced nephrotoxicity is being addressed in the generation of 3D cell culture models because these models exhibit features that are closer to the physiological conditions (Nickerson et al., 2007; Xia et al., 2014), and they are more realistic for translating the study findings for *in vivo* applications (Ravi et al., 2015). The generation of 3D PTC cultures as suitable model systems includes the incorporation of advanced biocompatible materials or functionalized biopolymer hydrogels as matrices in combination with highly differentiated renal cells. These new technologies are expected to revolutionize our ability to understand and predict clinically relevant renal responses for their application in kidney disease.

1.3. Engineered renal models for reducing animal studies

To study nephropharmacology the models currently applied include animal models (Gautier et al., 2010) and PTC monolayers (2D) (Chen et al., 1990). Drug testing studies and toxicological screenings use different animal species like mice, rats, hamsters,

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rabbits, fishes (zebrafish, trout), birds (mainly chicken), guinea pigs, amphibians (xenopus frogs), primates, dogs, cats, *etc.* The number of animals used in research every year has gone up with the advances in medical technology (Doke and Dhawale, 2015). Aside from ethical considerations, the use of animals in pharmacological preclinical testing is very time consuming, laborious and expensive (Badyal and Desai, 2014). These disadvantages have forced researchers to find new alternatives to decrease the time and the money involved in the studies and, of course, to decrease the number of animals used. Russell and Burch have defined these alternatives by three R's – Reduction, Refinement and Replacement (Arora et al., 2011). These alternative strategies include a big variety of new *in vitro* techniques, such as 3D cell cultures.

A new drug can not be used in patients until it has been extensively tested in animals, but the new alternative methods can help to reduce the number of animals required for nephro-pharmacological studies. The major benefits of 2D cell culture models are their simplicity, their compatibility with high-throughput drug screenings and their relatively low cost (Wu et al., 2009). However, in conventional 2D cell culture, cells spread mainly in horizontal direction, resulting in flattened cells that easily dedifferentiate and, as a consequence, are less physiologically relevant compared to 3D cell culture (Ferrell et al., 2010; Jang et al., 2013) (Table 1). The design of pharmacological studies based on 2D cell cultures could, therefore, be biased. Another disadvantage of these models is their incapacity of recapitulating the complexity of the *in vivo* environment (Bissell et al., 2002; Guo et al., 2008). To induce a toxic response in 2D cell culture models, usually higher doses over longer time periods are needed compared to *in vivo* and/or human toxicity responses (El Mouedden et al., 2000). In nephropharmacology, the use of 3D cell culture models of human origin reflects the physiological situation better. This allows detecting biomarkers indicative of nephrotoxicity *in vivo*, thus enabling the translation from *in vitro* to the *in vivo* situation. In support, it was demonstrated that a 3D cell culture system was more sensitive to nephrotoxic compounds than the same cells grown in 2D due to a preserved epithelial character. Additionally, long term studies revealed the utility of the 3D model for chronic toxicity studies as well (DesRochers et al., 2013). Various platforms applied in experimental nephropharmacological studies are discussed in the next chapter.

Table 1
Overview of conventional 2D cell culture *versus* 3D cell culture models.

	Advantages	Disadvantages
2D cell culture	– Simple model – Low-cost	– Flattened cells – Studies can be biased – Incapacity to mimic the physiological environment – Exposure of high dose over time to induce a toxic response
3D cell culture	– Improvement of physiological environment – Easy detection of biomarkers indicative for nephrotoxicity – It is a translational model from the *in vitro* to the *in vivo* situation – More sensitive to drug exposure	– Novel and more relevant 3D models are still under study – It will need time to adapt this technology to the labs and companies

1.4. Renal cells in use

Some of the most important morphological characteristics of PTC include a columnar shape epithelium with a cobblestone formation, the presence of a brush-border and the possibility to distinguish between an apical and a basolateral membrane because of cell polarization. Also, PTC are characterized by the differential expression and activity of specific membrane transporters and metabolizing enzymes. One of the most critical steps in the development of *in vitro* models to study nephrotoxicity is the ability to cultivate large numbers of cells with these specific phenotypical features. In this section, we are going to discuss the available sources of cells with defined renal phenotypes, with special focus on cells reproducing the proximal tubular phenotype.

1.4.1. Renal primary cell cultures

Renal primary cell cultures are defined as cells that have been freshly isolated from kidney tissue. In recent years, also isolating renal epithelial cells from human urine has successfully been achieved (Wilmer et al., 2010). Renal Primary cells closely mimic the physiological state of cells *in vivo* but the principal limitation of these cells is the process of dedifferentiation and the predetermined number of cell divisions before entering senescence. We already mentioned the proximal tubule to be the best studied segment from a clinical perspective. Thus, reports appear periodically describing new or improved methods to isolate and grow PTC (Sharpe and Dockrell, 2012; Valente et al., 2011; Vesey et al., 2009).

1.4.2. Immortalized cell lines of renal origin

The use of permanent cell lines began in the 1970s by renal and transport physiologists when they recognized that some of these cells retained a number of kidney-specific characteristics. More recently, continuous renal cell cultures have gained importance for investigating the pharmacology of potentially nephrotoxic xenobiotics, medicines and in general, to study nephropharmacology. These studies have revealed highly robust and reproducible proximal tubule specific functional results over prolonged culturing times (Nieskens et al., 2016).

Several permanent cell lines of renal origin have been established, in order to overcome the limitations of primary cells. The immortalization process is usually elicited by transfection and/or injection of Simian virus (SV40), papillomavirus (16E6/E7) genes, human Telomerase reverse transcriptase (hTERT) or transformation into primary cells of defined nephronal origin; this has been carried out with renal cells from various species, including human cells. Transformed cells acquire the ability to proliferate indefinitely; however, in most cases these cells had already suffered some dedifferentiation allowing them to grow under artificial conditions. Careful isolation, purification, and characterization have allowed for the generation of specific cell lines with adequate preservation of characteristic functional markers of defined nephron segments. In Table 2 the most widely used renal cell lines are represented, with special focus on cells reproducing proximal tubular phenotype.

In addition to human cell lines, animal-derived cell lines like MDCK, LLC-PK1, NRK-52 and OK have been extremely useful for *in vitro* research of normal and altered renal epithelial function because these cells have retained enough phenotypic parameters for studying specific characteristics or activities, and have lesser requirements and proliferate indefinitely, unlike primary culture.

2. Experimental models of renal cell cultures

The tubular structure is encased in the basement membrane (BM), a thin layer made of laminin, collagen IV, entactin/nidogen, and sulfated proteoglycans. As mentioned before, the renal tubule is a tiny tube subdivided in different segments, where the glomerular filtrate with wastes, extra fluid and other recyclable substances, like Na^+ and PO_4^{3-}, pass through.

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Table 2
Representation of the most widely used renal continuous cell lines.

Cell line	Specie	Presume Cell type origin	References
SGE-1	Wistar rat	Glomerulus	(Yamada et al., 1988)
NRK-52E	Norway rat	Proximal tubule	(de Larco and Todaro, 1978)
LLC-PK1	Hampshire pig	Proximal tubule	(Hull et al., 1976)
OK	American opossum	Proximal tubule	(Koyama et al., 1978)
MCT	Mouse	Proximal tubule	(Haverty et al., 1988)
JTC-12	Cynomolgus monkey	Proximal tubule	(Takuwa and Ogata, 1985)
HK-2	Human	Proximal tubule	(Ryan et al., 1994)
ciPTEC	Human	Proximal tubule	(Wilmer et al., 2010)
RPTEC	Human	Proximal tubule	(Wieser et al., 2008)
caki-2	Human	Renal carcinoma	(Fogh, 1978)
mTAL	Rabbit	Medullary thick ascending limb	(Scott, 1987)
MDCK	Dog	Distal tubule and collecting duct	(Gaush et al., 1966)
A6	Xenopus laevis	Distal tubule and collecting duct	(Rafferty and Sherwin, 1969)
PAP-HT25	Rabbit	Inner medullary epithelium	(Uchida et al., 1987)

Renal tubules are in contact with the vascular network, the interstitium and other renal tubules. All these relationships should be kept in mind if the goal is to understand and reproduce renal function. For this reason, recreation of the environment of the tubular structures is essential. Depending on the biological question that needs to be elucidated, multiple culture formats are available. The most relevant culture formats used in the field of nephropharmacology are discussed here in more detail.

2.1. Role of the extracellular matrix

In native kidneys, cells are embedded in a complex extracellular matrix (ECM). The ECM is a very dynamic and highly charged structure and it plays a very important role as an active component in cell signaling, support, morphogenesis, repair and regeneration (Bosman and Stamenkovic, 2003). In addition to its mechanical support function, the ECM harbors essential growth factors and signaling molecules important for tissue organization and function. The importance of cell-ECM interactions in driving differentiation towards a particular phenotype is well described (Lelongt and Ronco, 2003). An example of these interactions is found in the Human Kidney-2 (HK2) cell line, which showed an improved proximal tubular phenotype when cells were cultured on micro-scaffolds obtained by decellularizing 300 μm fragments of renal stroma (Finesilver et al., 2014).

The ECM is composed of basement membrane (BM) and the stromal matrix (SM): The BM is a sheet-like scaffold and comprises fibronectin, proteoglycans, laminin and collagen IV and it provides a number of physical and chemical interactions that cells need for proper self-recognition and differentiation. BM plays important roles in the kidney, illustrated by the fact that defects in renal BM are associated with kidney malfunction (Miner, 1999; Timpl, 1996). Obviously, renal cells recognize the roughness and hardness of the BM in the same way than other tissues (Kim et al., 2012). These properties were used in attempts to recreate artificial ECM substrates (Nur et al., 2006; Schindler et al., 2005) where it was appreciated that the topology offered by the polymeric structures can actually be more important than the bioactive signals they provide (Kim et al., 2014; le Digabel et al., 2010; Sciancalepore et al., 2014). The SM is composed of collagen I, proteoglycans and glycosaminoglycans, which form fibrous structures providing the major structural support of the ECM. The SM is responsible for holding together nephrons, blood vessels and other elements from the kidney parenchyma (Kuraitis et al., 2012). Integrins, transmembrane receptors located in the PTC play an important role as the mediators in the cell-ECM adhesion and signaling (Kanwar et al., 2004). After understanding the role of ECM in cell adhesion, structure and function, it is essential to incorporate its components in a 3D model.

2.2. Two-and-a-half-dimensional renal cell culture on ECM-coated surfaces

A drip culture is a cell culture format reflecting 2.5D, where the cells grow on top of an ECM and the growth medium of cells contains diluted ECM proteins. Although with 2.5D cultures, cells are in contact with medium and this situation does not recreate the *in vivo* environment, the advantage of this type of culture is the induction of a more physiological architecture than conventional 2D cultures (Chen et al., 1990; Shamir and Ewald, 2014). Some of the most relevant applications of this cell culture format are for imaging and antibody staining. Also, it is a great system for studying epithelial acinar formation in MDCK cells (Madin–Darby canine kidney) (Shamir and Ewald, 2014). Prashanth Asuri et al. created a 2.5D platform to study migration. The platform consisted of a layer of alginate on top of a monolayer of cells grown on tissue culture polystyrene and they used Human Embryonic Kidney (HEK) 293T cell lines to test the effect of stiffness and mechanotransdutive signaling on adhesion-independent cell migration. This platform provides a breakthrough for the study of new drugs with anti-metastic properties in cancer research (Pebworth et al., 2014).

2.3. Two-and-a-half-dimensional renal cell culture in transwell devices

A Transwell is a membrane insert used for cell cultures, which ensures the formation of a compartimentalized system, allowing the cells to polarize. This also offers the possibility to work with co-cultures in independent compartments that communicate through the release of signaling molecules. However, due to the high costs and the fact that working with Transwells is laborious, these devices are extensively used in industry but they have not been widely adopted in academia. Brown et al. (2008) worked with tubule cells grown on Transwells as a robust, polarized primary cell culture model of the human proximal tubule to study the mechanisms and regulation of xenobiotic transporters. Tubule cells were isolated and grown on the inserts for up to twelve days. The expression of key transport proteins was assessed at both the mRNA and protein expression levels. This study helped to understand the contribution of individual transporters in the basolateral or apical side to the overall renal handling of a drug molecule (Brown et al., 2008; Schophuizen et al., 2015).

2.4. Three-dimensional renal cell culture on ECM-coated surfaces for bioartificial kidney applications

The increasing incidence of end-stage renal disease, a shortage of kidney organ donors, and the significant impact on patient's life of current dialysis and hemofiltration techniques, generates an urgent need for alternative renal replacement therapies. One of the most actively pursued potential renal replacement therapies in the last years is the bioartificial kidney (BAK), a cell therapy based on *in vitro* culture of renal cells. It consists of the combination of a hemofilter in series with a bioreactor unit containing renal PTC, termed a renal assist device (RAD). Cells are seeded and grown as a confluent monolayer in the lumen of hollow fibers. The hemofiltrate is passed through the lumen, and the blood through the

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space between the fibers. The goal is that cells reabsorb biologically relevant substances from the filtrate, secrete toxins into the filtrate, and produce metabolic and endocrine functions of renal epithelia. In this way, the hemofilter would provide the glomerular function while the cell-based cartridge will be delivering those functions of the tubular portion in the nephron (Jansen et al., 2014; Tasnim et al., 2010).

The BAK concept started by Aebischer et al. (1987). In the next years, devices improved and became more sophisticated. BAKs received further push in 1999, when the group of Dr. Humes utilized porcine renal proximal tubule cells (LLC-PK1) cultured on semipermeable polysulfone hollow fiber membranes on which extracellular matrix, pronectin-L, was layered to enhance cell attachment and growth (Humes et al., 1999). This group reported later on the safety and efficacy of BAKs use in patients with acute renal failure (Humes et al., 2004). The device demonstrated essential renal functions (including excretory, metabolic and endocrine pathways) and immunomodulatory activities. However, phase II trials had to be interrupted due to undesired adverse effects and technical issues, despite some clinical improvement had been observed through phase I trials. The limitations presented in the clinical trial, linked with the impossibility of implementation in the public healthcare on short-term and aspects of BAK development related to restricted cell sources, are forcing the scientific community to search for new solutions.

In 2012, the group of Dr. Humes introduced the Bioartificial Renal Epithelial Cell System (BRECS). The BRECS is the first all-in-one culture vessel, cryopreservation storage device, and cell therapy system. This is a regular cell bioreactor designed to be fully cryopreserved at -80 °C or -140 °C. The BRECS was designed to maintain a dense population of adult human renal epithelial cells grown on porous, niobium-coated carbon disks within the system. After the cells reach an optimal density, the BRECS can be cryopreserved, transported, and stored at a clinical site, thereby alleviating many practical limitations previously encountered by cell-based therapies. This design represented the newest technology in conservation of human renal epithelial cells (Buffington et al., 2012; Jansen et al., 2014). A recent breakthrough was achieved by culturing cells on hollow fiber membranes that demonstrate maintained morphology and functionality (Jansen et al., 2015, 2016). This concept needs to be upscaled, as discussed by Chevtchik et al. in this Special Issue of European Journal of Pharmacology.

2.5. Three-dimensional renal cell culture in hydrogel

Culture systems that better mimic the biological milieu are needed to bridge the gap between conventional cultures and complex native *in vivo* environments. Hydrogels are good tools for getting this goal. A hydrogel is a biocompatible polymer network with high water content and with physical properties that closely mimic the natural ECM. This ability to swell under biological conditions makes them an ideal class of materials for biomedical applications, such as drug delivery and tissue engineering (Caliari and Burdick, 2016; Lee et al., 2008; Lutolf, 2009). A renal cell 3D culture system consists of cells embedded in an ECM gel generated by mixing the renal cells with a liquid ECM matrix at the time of seeding (Desrochers et al., 2014; Shamir and Ewald, 2014). These gels then polymerize based on physical (*e.g.* temperature or light) or chemical (*e.g.* pH or ionic strength) stimuli (Ahmed, 2015). Currently, hydrogels can be classified based on their origin: natural or synthetic. There are several types of hydrogels, ranging from simple and inert such as alginate, to complex and highly cell active such as the commercially available and widely applied Matrigel (Table 3). Also, hydrogels can be classified for the type of crosslink and in this classification, for which two categories of hydrogels have been distinguished: permanent/chemical gels or reversible/physical gels. The first category is called 'permanent' or 'chemical' gels. Here, the gels are covalently cross-linked by replacing a hydrogen bond by a stronger and stable covalent bonds networks. They can reach an equilibrium swelling state depending on the crosslink density and the polymer-water interaction parameter. The second category is called 'reversible' or 'physical' gels because molecular entanglements and/or secondary forces, including ionic, hydrogen bonding or hydrophobic interactions, hold the networks together. In physically cross-linked gels, the dissolution is prevented by physical interactions between different polymer chains (Hennink and van Nostrum, 2002). These interactions are reversible and can be disrupted by application of stress or changes in physical conditions. The last classification we are going to mention in this manuscript is based on physical structural features of the hydrogels, and they can be classified as amorphous hydrogels, semicrystalline hydrogels, and hydrogen-bonded or complexation structures. In amorphous hydrogels, the macromolecular chains are arranged randomly. Semicrystalline hydrogels are characterized by dense regions of ordered macromolecular chains. Finally, hydrogen bonds and complexation structures may be responsible for the 3D structure formed. These properties make the hydrogels most versatile and prevalent 3D models for *in vitro* studies (Ahearne, 2014).

Tubules generated by inclusion of NKi-2, an immortalized proximal tubular cell line, in collagen I–Matrigel gels have been compared to 2D cultures as cytotoxicity models to study the toxic effects of cisplatin, doxorubucin and gentamicin. Lactate dehydrogenase (LDH) secretion and the use of two predictive kidney injury markers, KIM-1 and NGAL were used as the main readout and revealed a difference in the overall toxicity and timing of toxic events between 2D and 3D culture conditions of the same cell line. The NKi-2 cells from 3D cultures exhibited better expression of functional markers and higher sensitivity to the nephrotoxicants than the 2D cultures. These results could be explained by increased drug uptake, metabolism, and toxicity due to enhanced epithelial characteristics (DesRochers et al., 2013). Another relevant study in this field is the 96-well format 3D culture model of proximal tubule cells for studying nephrotoxicity developed by Astashkina and colleagues. The model is based on culturing primary mouse PTC inside a hyaluronic acid matrix. Under these conditions, the cells form tubular structures and express specific markers for at least 2 weeks. When compared to immortalized cells LLC-PK1 and HEK-1 growing as monolayers directly on plastic, the proximal tubule 3D system exhibited a more clinically relevant response to nephrotoxicants (Astashkina et al., 2012). This model has recently been used to evaluate nanoparticle nephrotoxicity as well (Astashkina et al., 2014).

3. Other nephropharmacological *in vitro* models

The formation of a complex architecture *in vitro* and the incorporation of factors such as shear stress forces due to luminal fluid flow represent new 3D kidney model alternatives to apply in the area of nephropharmacology. These include decellularized kidney as native ECM scaffolds, kidney on a chip technology and 3D bioprinting techniques, which will be discussed briefly in this section and in more detail in reviews included in this Special Issue of European Journal of Pharmacology.

3.1. Decellularized kidney

Recent developments within the field of regenerative medicine also include the generation of bioscaffolds through organ decellularization. In this process the ECM is isolated from a tissue by

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Table 3
Characteristics of the main hydrogels commercially available.

Origen	Advantages	Disadvantages	Examples	References
Natural	– Biocompatible – Bioactive – Presence of various endogenous factors (it can help for supporting viability, proliferation, function and development of many cell types)	– May contain biological pathogens – Batch-to-batch variability in composition – Low mechanical strength	– Collagen – Laminin – Fibrin – Hyaluronic acid – Chitosan – Matrigel	(DesRochers et al., 2013; Shimazu et al., 2001)
Synthetic	– Biologically inert – Highly reproducible – Simple to process and manufacture. – They can be customized with specific peptide sequences to improve cell behaviors	– Can include toxic substances – Low biodegradability – Inherent bioactive properties are absent	– Poly(ethylene glycol) – Poly(vinyl alcohol) – Poly(2-hydroxy ethyl methacrylate)	(Chung et al., 2008; Tsurkan et al., 2013)

removing its inhabiting cells and leaving a native ECM scaffold. Various researchers demonstrated that decellularized kidneys from animals or human can be used as 3D biological scaffolds. The decellularization process conserves the mechanical and biological properties of the ECM, generating a template that can maintain natural stromal architecture and some residual molecules and thus may promote attachment, differentiation and proliferation of newly grafted cells (Hodde et al., 2002; Hodde et al., 2001). Eventually, the regenerated tissue can be used as a transplantable organ. Since the kidney has one of the most complex architectures of the body, generating an efficient decellularization method that preserves the vascular networks and parenchymal anatomy of the native kidney has been a strongly pursued objective in regenerative medicine. The advances in this field have allowed for the development of different techniques to decellularize rodents (Ross et al., 2012), porcine (Sullivan et al., 2012), rhesus monkey (Nakayama et al., 2011) and human (Song et al., 2013) kidneys. Next to being a source for organ transplantation, decellularized-recellularized kidneys can also be used as a model to study the interaction of drugs affecting tissue failure and enhancing repair mechanisms. Currently, the principal limitation to obtain functional recellularized kidneys is the complexity of the organ, requiring advanced bioengineering.

For recellularization, knowledge can be obtained from the rapidly evolving field of mini-organs, called kidney organoids (Morizane et al., 2015; O'Neill and Ricardo, 2013; Takasato et al., 2015), which is addressed in two dedicated reviews within this Special Issue. These mini-organs form more sophisticated models to fill the gap between *in vitro* and *in vivo* understanding of functional kidney development and repair (Davies, 2015).

3.2. Bioprinting

Three dimensional kidney bioprinting is a new technology with the goal of developing functional full size kidneys. This new emerging technology is based on the use of computers and modified printers-based technology, where biomaterials chosen to create *de novo* full size kidney are used to print layer-by-layer specific biological materials, with spatial control of the placement of functional components (Murphy and Atala, 2014; Peloso et al., 2015). The technology is based on three central approaches: *i.* biomimicry, which involves the production of identical reproductions of the cellular and extracellular components of a tissue or organ (Ingber et al., 2006); *ii.* autonomous self-assembly, referring to the capacity of cells to drive the histogenesis, directing the localization, composition, functional and structural properties of the tissue (Derby, 2012); and *iii.* mini-tissue building blocks. Currently, researchers do not have the capacity for building a complex and large 3D kidney; therefore, the most relevant studies published have focused on kidney 3D bioprinting on a small scale with the generation of 'mini-tissue' building blocks. This approach includes two strategies: the first one is the self- assembling cell spheres. Cell spheres are assembled into a macro-tissue using biologically inspired design and organization. The Davies lab published a relevant publication using this strategy. This group used renal stem cells that re-aggregates and self-assemble to form an organized kidney mini-tissue (Unbekandt and Davies, 2010). The second strategy is to get the reproduction of a tissue unit and then self-assemble it into a functional macro-tissue. The technology of this approach is being used for the generation of functional microfluidic devices (Jang and Suh, 2010). The goal of 3D bioprinting is the creation of *de novo* kidneys, but other applications are well feasible and include the development of high-throughput 3D-bioprinted tissue models for research, such as in drug efficacy and safety screenings.

3.3. Microfluidic devices

To recreate the physiological environment found *in vivo* in 3D cell models, the addition of bioreactor systems is essential because it will allow a continuous fluid flow on the cells. This new approach, possible with the use of microfluidic devices such as kidney microchips, is discussed in more detail by Nieskens and Wilmer in this Special Issue. Microchip technology is defined as a cell culture model in a system with a micrometer scale that incorporates important features like dimensional and morphological relevance, flow shear stress, mechanical strain and co-culture capabilities, among others (Wilmer et al., 2016). A variety of cells of renal origin have been grown in microfluidic devices: *e.g.* primary human proximal tubule (Ferrell et al., 2010), primary inner medullary collecting duct (Jang and Suh, 2010) and HK-2 cells (Wei et al., 2012). The design of the microchip depends on the desired purpose. One parameter necessary to design a microchip is the geometry of the system. The material selected for the fabrication could be a limitation, for this reason it is necessary to choose materials very carefully. For instance, when used in combination with live imaging, materials should have optimal optical properties to avoid auto-fluorescence. During the design phase, it is also necessary to consider whether the available microscope set up is compatible with microfluidic devices (Sánchez-Romero and Gimenez, 2015).

One advantage of the microfluidic devices is that it saves expensive reagents because of the small volumes running through the micro-scaled chambers. A large part of the work with microfluidic devices demonstrated that this new technology is widely applicable in biomedical research, so this technology is highly valuable for studying renal physiology and pathology. In the nephropharmacological area, Choucha-Snouber et al. (2013) developed a microfluidic kidney model to study the nephrotoxic effects of ifosfamide. Ifosfamide is a drug metabolized by the liver into a bioactive and nephrotoxic compound. Using a liver micro-device directly linked to a kidney device, they demonstrated the

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interaction between the two organ systems, mimicking the sequence of events. The kidney cell toxicity was apparent only when the liver metabolized ifosfamide and the metabolite was subsequently perfused through the kidney device, but not when the order of exposure was reversed.

Jang and colleagues published another relevant publication (Jang et al., 2013). They compared in human primary proximal tubule cells the effect of cisplatin exposure for 24 h when cultured in a microfluidic device *versus* a static culture. The results revealed that in control settings, cells under fluid flow were healthier compared to static cultured cells, and also demonstrated a more representative toxicity response. Furthermore, the study showed that the cells under fluid flow were almost completely protected from a toxic response to cisplatin when an OCT-2 inhibitor was administered at the same time as the drug, indicating that the technology can be applied to study drug-drug interactions and drug therapy strategies. Finally, Jang et al. (2013) showed that the cells under fluid flow performed better at recovering from cisplatin toxicity than the cells in static culture, suggesting a role for shear-stress in renal repair. In addition to renal toxicology, efficient cell differentiation (Zhou et al., 2014) and kidney stone formation (Wei et al., 2012) have been addressed by using microfluidic devices.

4. Concluding remarks and future directions in *in vitro* models to study nephropharmacology

Applications of 3D technologies to renal related research have demonstrated to support the microenvironment crucial for translational science. The combination of these technologies will allow creating innovative methods to analyze the mechanisms of action and interactions of drugs in the kidney. Technologies like kidney-scaffolds, organoids and microfluidic cultures can be made compatible with high-throughput screening. This is attractive for pharmaceutical industries aiming at developing safer drugs. However, it should be noted that the new 3D culture technologies are yet immature and currently not easily implemented into academy or commercial laboratories. If we want to include fluid shear stress in the experimental designs, the use of specific equipment like incubators with integrated perfusion systems will be necessary. The implementation of all these systems in the lab harbors the risk of culture infections and in renal cell-related research, the use of antibiotics in cultures is preferably avoided as many compounds exert nephrotoxicity (Fanos and Cataldi, 2001; Nigam et al., 2015). Despite these limitations, the promise to obtain a better differentiation, enriched cell–cell interactions and recreation of the *in vivo* physiology will result in more representative experimentation in the nephrotoxicity field.

In conclusion, it should to be noted that 2D cell models have made good contributions to understanding overall renal drug handling. However, 3D cell culture models are better suited than the traditional 2D cell culture model. Currently, 3D cell culture models represent a great promise for applications in drug discovery due to improved cell-ECM, and cell-cell interactions, forming structures that resemble the architecture and possess the physiology of *in vivo* renal tissue.

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