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Engineered renal tissue as a potential platform for pharmacokinetic and nephrotoxicity testing

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Abstract

Pharmacology and regenerative medicine interact in two ways. One is the use of drugs to promote tissue regeneration. The other, less obvious but with great potential, is the use of techniques developed for regenerative medicine to engineer realistic human organoids for drug screening. This review focuses on testing for nephrotoxicity, often a problem with drugs and poorly predicted in animals. Current human-based screens mainly use proximal tubule cells growing in 2D monolayers. Realism might be improved by collagen-based culture systems that encourage proximal tubule cells to grow as tubules. More realistic would be a recently developed technique for engineering functioning ‘mini-kidneys’ from suspensions of stem cells, a technique that works in mouse but that could also be applied to humans.

Introduction: why we need human-based *in vitro* models for renal physiology and nephrotoxicity

It is increasingly recognised that animal testing of drugs is, on average, only poorly predictive of side effects that appear in humans [1]. There are a number of possible reasons for this, including animal – human differences in the intended drug receptor, unexpected human-only cross reactions with alternative receptors and significant differences in drug metabolism and elimination. For many drugs, the liver is the most important centre of metabolism and its cells often the main victims of toxic metabolites. For this reason, there is intense interest in making culture models of human liver, with metabolism as realistic as possible. Careful comparison of 2D and 3D culture systems shows the realism of 3D models to be superior for predicting whole-body toxicity [2]. Some approaches even aim to derive liver models from a patient’s own cells via induced pluripotent[s3] stem (iPS) cells [3], which will allow testing of drug metabolism in humans of different genotypes and a significant move towards patient-specific treatment.

The liver is not, however, the only major site of drug metabolism and some molecules are metabolised by pathways that operate in the kidney, generally in the cells of the proximal convoluted tubule [4]. These cells acquire the drug and/or its metabolites via two principle routes, depending on the molecule involved (Figure 1). One route is direct from the extracellular fluids in the tissues. Proximal tubule cells have active uptake systems

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for organic anions and cations, either from the basal or apical sides of the cell (depending on the molecule in question) that ‘push’ these molecules even against a concentration gradient: from the cytoplasm, molecules leave either passively through any channels that will carry them or use active apical export systems such as P-glycoprotein. Proximal tubule cells also have very active endocytosis systems that recover larger molecules such as small proteins that have passed through the glomerular filter into the urinary space: generally, these bind to a complex of megalin (also known as LRP2 [s4]and gp330) and cubulin, two interacting large apical membrane proteins expressed by proximal tubule cells. They[s5] are endocytosed with them [5,6].

The transport systems described above are important to pharmacologists in two main ways. First, they are of obvious relevance to pharmacokinetics and can be the main route of elimination [4]. This is one of the reasons why renal dysfunction can have such a dramatic effect on plasma drug concentrations, although more subtle interactions between a diseased kidney and the body can also effect drugs normally eliminated elsewhere [7]. Second, they can be the cause of serious renal damage. The ‘push in, drift out’ mechanism that applies to some molecules is safe for those for which an efficient export route is available but, where such a route is not available, the cytoplasmic concentration can build to toxic levels very quickly. The same is true where an imported molecule is metabolised into a toxin that has no efficient exit route. The megalin-driven endocytic mechanism can also cause dangerous accumulation of small molecules that happen to bind to the megalin–cubulin complex and become endocytosed. An important example is the aminoglycoside antibiotic gentamicin [8]. It is a sobering statistic that, in a study of 100 patients conducted in a renal unit, 20% of adult kidney disease cases were iatrogenic [9], caused mostly by drugs or their metabolites, whereas another study claimed that about 1 in 200 patients hospitalised (from all causes) suffers a hospital-acquired renal injury [10]. Occasionally this damage is unforeseen but frequently it is simply an inevitable risk of treating an even greater threat, such as an infection, by a drug known to be nephrotoxic. There is a significant research effort to find less nephrotoxic drugs and to find molecules that can be used in combination therapy, for example to prevent aminoglycoside antibiotics being endocytosed by the megalin – cubulin system, and thus render them harmless to the kidney [11]: statins show promise in this application [12]. Not all patients show the same nephrosensitivity to drugs, and nor do isolates of their cells [13]: it would therefore be useful to find ways (e.g. genetic tests) to identify those who are at most risk. In summary, for the purposes of predicting renal drug metabolism, renal drug excretion and unwanted nephrotoxicity, for development of renoprotective combination therapies and for developing ways to identify patients most at risk, there is an urgent need for realistic culture models of the human kidney, especially the proximal tubule.

Existing culture models

As with so many other systems [14], culture models exist in a range of complexities, with the usual trade-off between realism and ease of use. At the simplest end of the spectrum are 2D cell cultures of single cell types in conventional culture flasks, dishes or wells. Slightly more realistic are similar cultures made as sealed monolayers on filters in well-inserts, for which the medium used for bathing the apical (urinary) side can be made different from

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that used for bathing the basal (tissue) side. Three-dimensional cultures of single cell types, in gels or in 'organs-on-a-chip', represent the highest level of complexity in current use: potential future developments, using techniques developed for regenerative medicine, are described later.

The HK2 cell line

The HK2 cell line is a human proximal tubule cell line, derived by retroviral transformation [15]. It has been used widely in studies of transport and nephrotoxocity (searching PubMed with 'HK-2 proximal' yields hundreds of such studies, far too many to be cited here[s6]). Careful examination of the transport properties of these cells suggests, however, that they are less than faithful in representing the transport systems of proximal tubule cells *in vivo* and that they are therefore of limited use as a model [16]. The findings that have been discovered using them and thus extrapolated to the *in vivo* situation should therefore be treated with great caution.

Primary culture of human proximal tubule cells

Living human proximal tubule cells can be obtained directly from a kidney via biopsy, nephrectomy or post-mortem. Biopsies yield only small numbers. Nephrectomies yield more but nephrectomies are done for a reason and the donor kidney can be damaged, infiltrated with a tumour or both. Fresh post-mortem kidneys are sometimes available as transplant-ready but, for one reason or another, are unable to be used for the planned operation: these tend to be the highest quality source but their supply is not predictable.

Protocols that consist of dissection, enzyme treatment, sieving, density gradient centrifugation and sorting for the proximal marker leucine aminopeptidase yield almost pure cultures of proximal tubule cells [17]. These can be cultured on the membranes of Transwell® inserts, which allow access to the apical [s7]and basal domains. Under such conditions, they form a monolayer with an electrical resistance typical of the proximal tubule, they continue to express transport channels and they transport drugs and test substances efficiently [17]. In conjunction with inhibitors of specific channels, uptake and efflux pumps, they have been used to identify the routes by which specific small molecules enter and leave human proximal tubule cells [18]. In this respect, they could be an excellent system for high-throughput studies. There are, however, problems with these cells in that they can be used only for a week or two, and only if left as direct primary cultures. If the cells are passaged, they quickly lose their physiological properties and become very poor models of the intact kidney. Given the restrictions in their supply, this is a serious limitation of their use in high-throughout systems although they remain excellent for low-throughput studies of specific transport systems.

Conditionally immortal proximal tubule cells

One approach to the problem of culturing cells with intrinsically low mitotic activity is to transfect them with an immortalising construct that drives their replication under 'permissive' conditions, such as low temperature, and becomes inactive again under nonpermissive conditions. The hope is that, once returned to nonpermissive conditions after a period of population expansion, the cells return to what they were doing originally

[19]. This idea has been applied to proximal tubule cells, most notably by the production of ‘conditionally immortal proximal tubule epithelial cells’, abbreviated to ciPTEC, from proximal cells recovered from urine. Immortalisation is in this case performed using temperature-sensitive SV40 large T antigen and human telomerase [20].

ciPTEC can multiply well in permissive conditions and, when returned to 37°C, show fairly normal transport properties. For example, they show expression of brush-border-associated enzymes, saturable endocytosis of labelled albumin, sodium-dependent uptake of phosphate, OCT2-(SLC22A2-)dependent basolateral uptake of organic cations and an active P-glycoprotein-mediated efflux transport system [20]. The cells show sensitivity to gentamicin[s8], and have been used as a model to screen for treatments that encourage regeneration after gentamicin exposure [21]. Given the accessible nature of the starting material, it is feasible to generate ciPTEC lines from different patients to investigate the impact of genetics, or perhaps chronic disease, on proximal tubule cell biology. This production of patient-specific ciPTECs has, for example, been done for a study of Dent disease, which features a mutated chloride/proton antiporter [22].

Three-dimensional culture of proximal tubule cells

Proximal tubule cells do not normally exist as flat sheets, and one step towards further realism is to culture them so that they form tubules. An early example of this was provided by Chang and colleagues, who explanted intact pieces of human renal cortex on collagen gels and found that tubules survived and invaded the gel structure [23]. The culture was, by the nature of the starting material, highly heterogenous. More recently, primary cultures of urine-derived proximal tubule cells have been placed in ‘collagen sandwich’ culture, where they form tubules rather than sheets [24]. The adluminal surfaces of the cells sport microvilli, just as they should *in vivo*. This system has not, to this author’ s knowledge, yet been used in studies of nephrotoxicity but it can capture certain aspects (e.g. effects of concentration in the restricted lumen) more realistically than flat sheets will.

Kidneys-on-a-chip

An alternative to allowing proximal tubule cells to make their own tubule in collagen gel cultures is to pattern fine-channels in microfluidic devices that can be attached to fluid pumps and sinks, and then to seed these channels with cells. Jang and colleagues have recently built such a device that also features a semi-permeable membrane to allow separate fluid access to the apical and basal domains [25]. In this system, the presence of pumped fluid flow induced the proximal cells to acquire a much more normal columnar shape than is seen in flat monolayers, with an accompanying increase in channel protein expression and in transepithelial transport. When used to assess the toxicity of known nephrotoxins, cells under fluid flow showed responses claimed to be much more representative of those seen *in vivo* than when flow was absent. The reproducible nature of microfluidic devices, and the possibility of flushing them, makes them well suited to high-throughput analyses.

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Moving to ‘mini-organs’ by harnessing techniques of regenerative medicine

Even the ‘kidneys-on-a-chip’ described above, arguably the most advanced of current technologies, are a long way from the complexity of a complete organ, although recapitulation of some organ-level functions has been achieved and more could be feasible soon. At the moment, renal-organs-on-a-chip might be useful for researching cell specific transport or metabolic processes but are less helpful in studies of toxicity, where interactions with cell types other than the proximal tubule could be very important. What is needed is some way to culture small-scale functional human kidneys.

The rising incidence of end-stage renal disease and the worsening shortage of transplantable kidneys have encouraged a search for methods to engineer human kidneys *de novo* from stem cells. Building a full-sized adult organ is currently well beyond our capabilities but building an engineered ‘mini-kidney’, with a full range of cell types and appropriate physiological functions, might be much nearer. Although this would not be much direct use in transplantation, it could be very useful as an advanced culture system for nephrotoxicity testing.

Working with mouse renogenic stem cells (isolated directly from the part of the embryo that is about to make a kidney), we have developed a method that can turn a simple suspension of isolated cells into a small mini-kidney (Figure 2). This contains a branched urinary collecting duct system that defines the shape of the organ. It also contains excretory nephrons, with their Bowman’ s capsules, proximal tubules, loops of Henle and distal tubules, properly formed and arranged organotypically[s9] and expressing the usual marker genes. The neprhons connect to the collecting duct tree [26 – 28]. When grafted to a source of blood vessels, such as the chorioallantoic membrane of a developing chick egg, the kidney encourages ingrowth of vessels and glomeruli form [29]. When grafted into a host mammal, the kidney rudiments connect to the blood system and filter blood [30].

We are still in the process of comparing the transport activities of the proximal tubules of these kidneys with those activities in intact adult organs, but preliminary results are encouraging. If the transport systems are comparable, then these engineered mini-kidneys would be a promising, realistic culture system. How difficult would it be to make a human version? Two advances would need to be made. The first remains in the murine system, and is the production of renogenic stem cells from mouse iPS cell [rather than by dissection of an embryo, something not feasible for humans for obvious ethical and practical (staging) reasons]. A few reports have described methods for making renogenic cells from embryonic stem cells, but so far with efficiencies much lower than would be practical [31 – 34]. These methods need to be improved. If a working system can be made in mouse then it could be applied to human iPS cells in the hope that it works at least efficiently enough to allow later optimisation in this other species. In principle, patient-specific cells could be used. In all cases, careful assessment of physiological function will need to be made, for example by studying transproximal transport of fluorescently tagged organic anions and cations, glomerular filtration of fluorescently tagged dextrans from endothelial to urinary compartments and resorption of fluorescently tagged proteins. It can be expected that these models will represent natural physiology only imperfectly, but it might still be possible to

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study the effects on them of drugs with known effects on normal human kidneys, and to ‘ calibrate’ the system, relating the magnitude of changes seen in engineered systems with the changes in the real thing. In this way, reasonable predictive value could be obtained even when precise physiological parameters differ between test systems and real people. The problem with the engineered mini-kidney approach is that it is complex and difficult to automate. It will probably therefore be used only at the last stage of a hierarchical screen, for only those drugs that look promising in the simpler protocols.

Concluding remarks

The kidney is by no means the only organ that tissue engineers are trying to create from stem cells. Endocrine pancreas and liver are two other examples that are receiving a great deal of attention. In all cases, the path from current methods to clinical application in transplant is long but, again in all cases, even the crude and small organoids that can be produced by current technology could be very useful for screening purposes. It is commonplace for pharmacologists to engage with specialists in regenerative medicine with a view to finding ways in which pharmacology can help regeneration. Now could be a good time to reverse the conversation, and to notice the ways in which even modest achievements in regenerative medicine could help pharmacology.

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Teaser[s2]

Human cell based nephrotoxicity screens typically use 2D proximal tubule cell cultures. A kidney tissue engineering technique, developed for regenerative medicine, has potential to deliver much more realistic assays.

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Highlights

- There is an urgent need for better *in vitro* nephrotoxicity screens
- 2D cell proximal cell cultures exist but are imperfect
- A new 3D kidney tissue engineering technique has potential to deliver more realistic assays

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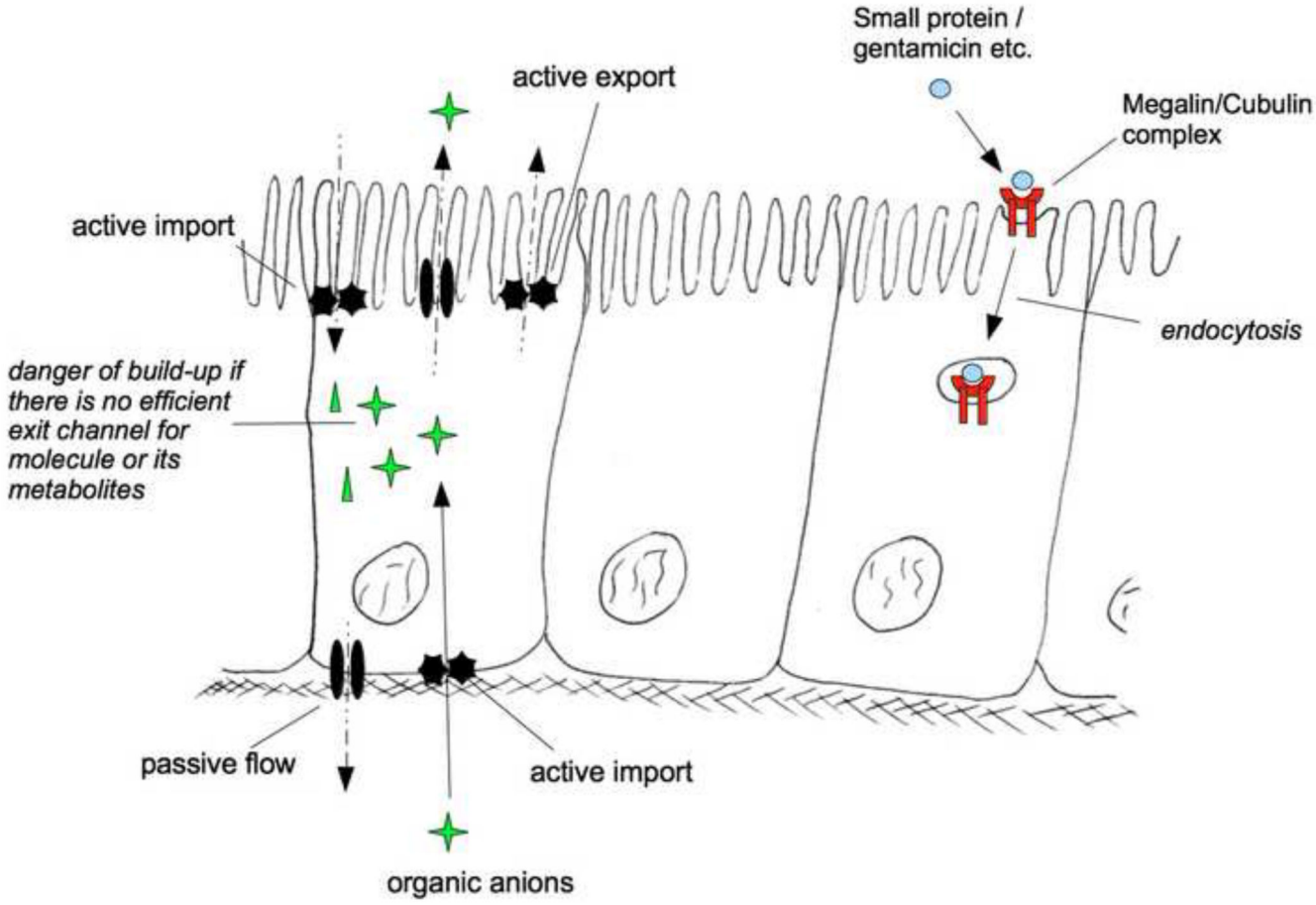

Figure[s10] 1.

Routes of uptake of drugs into cells of the renal proximal tubule (simplified to show only major paths). Organic anions and cations are transported across cell membranes by the proteins of the organic anion transporter (OAT) and organic cation transporter (OCT) (SLC22) family of transporters, which have differing (poly) specificities and different expression patterns on the apical and basal sides of cells, thus driving vectorial transport. These exchange import of organic anions for export of ketogluratate and similar molecules, the transmembrane gradient of which is established by other processes that are powered, ultimately, by the Na/K ATPase. The imported anions and cations exit either passively or via active exchangers such as P-glycoprotein. If no efficient exit route exists, either for the imported molecule or its metabolites, there is a risk the cytoplasmic concentrations will become toxic. Small proteins and other molecules, such as aminoglycosides, are recovered from the luminal space by endocytosis driven by the megalin – cubulin system. Some organic cations (such as choline) are also recovered from the lumen by various transport systems [35].

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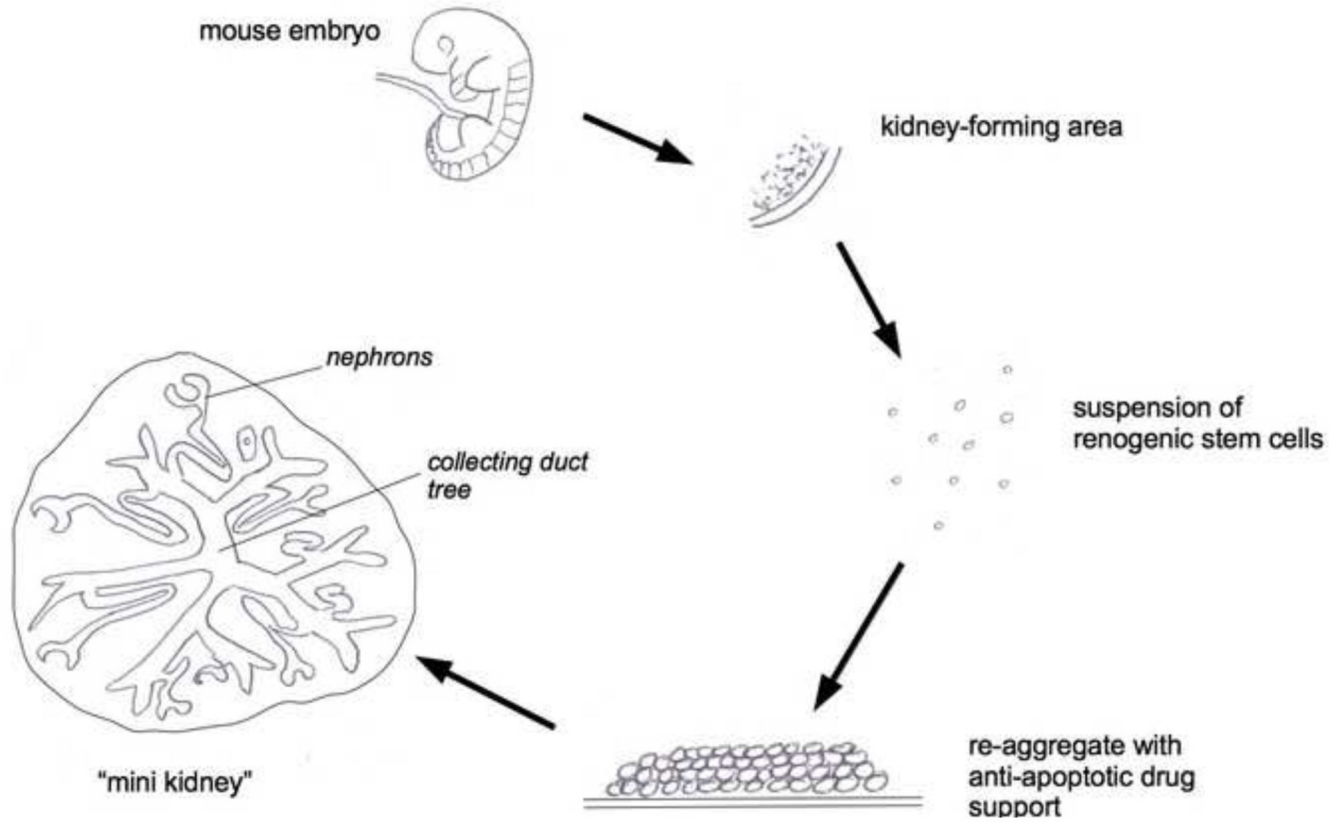

Figure[s11] 2.

Production of ‘ mini-kidney ‘ from mouse renogenic stem cells, which are re-aggregated in the temporary presence of Rho kinase (ROCK) inhibitors to protect them from apoptosis. The mini-kidneys can connect with vascular systems either *in ovo* or *in vivo*, and will filter tracer molecules from blood.