Phenotypic screening with 3D cell-based assays

Booij, T.H.

chapter 1

General introduction

Tijmen H. Booij

Challenges in drug research & development

Over the past decades, it has become clear that the increasing investments in pharmaceutical research and development (R&D) have not translated into an anticipated increase in approved new drugs. With new drugs being discovered at a steady rate by the pharmaceutical industry and the exponentially rising R&D costs, it is becoming more difficult to obtain a return of R&D investments and to fund new research. It is possible that the current R&D strategies are exhausted and that the only solution to this problem is a radical change towards more innovative strategies to improve success rates of new drugs.[1] Additionally, when older patents of important blockbuster drugs expire, the pharmaceutical industry will be required to address R&D productivity to remain viable.

As a brief summary of the steps in current drug discovery, novel drug candidates are typically discovered after the identification of a new drug target. Drug targets are often proteins that are aberrantly active or inactive in a pathophysiological process, and modulation of these targets, or their signalling cascade, could therefore be used to alleviate or reverse disease symptoms. Not surprisingly, the identification of new drug targets typically requires many years of intensive study of disease-associated cellular signalling pathways. After a disease target has been identified, potential drug candidates that bind to the target protein, often many thousands, can be synthesised and assayed on cell culture models for the disease. This pre-selection on cell culture models is required in order to preselect a small number of molecules for *in vivo* efficacy measurement and clinical development (figure 1).

This particular drug discovery workflow is target-based: it relies on known information of disease targets.[2] A drawback associated with this strategy is the assumption that modulation of a single protein target is sufficient for the alleviation of disease symptoms: many diseases are much more complex than this and require a broader targeting approach, which can for example be overcome by treatment with multiple drugs. Another consequence of this target-driven drug discovery is that only the most potent inhibitors or activators for certain potentially druggable targets will progress through drug development. While these molecules may be curative for a certain disease, many diseases do not require complete abolishing of one molecular target, but rather require fine-tuning of multiple target proteins, which is often the most challenging to achieve. Importantly, entirely abolishing a single disease target can also lead to drug side effects. Antineoplastic drugs (chemotherapeutics) are an example of this: while these drugs mostly interfere with cell division or promote programmed cell death and thereby inhibit tumour growth, they can also have similar effects on healthy cells, with side effects as a result.

In addition to the increasing R&D costs, high drug attrition rates pose a large challenge. Most drugs initially introduced in the drug development pipeline will never reach the clinical evaluation stage. However, even for drugs that enter clinical trials, the average success rate is only around 11%.[3] Because this phase in drug development is associated with the highest costs, it is essential for the pharmaceutical industry to

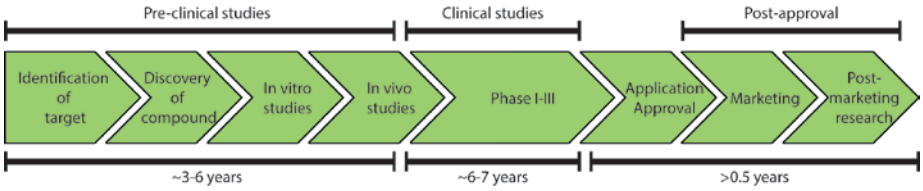

FIGURE 1 Schematic representation of the drug discovery pipeline resulting from the identification of druggable targets.

ensure that new drugs do not fail during this phase, or after the clinical trials. However, many drugs that are introduced in clinical trials suffer from lack of efficacy or toxicity that was not predicted.[4] Additionally, the success rate of clinical trials highly differs between therapeutic areas. For example, drugs for cardiovascular indications have an approximate 20% chance of success, while this is only 8% for drugs targeting diseases of the central nervous system.[3] These differences are likely, at least in part, attributable to our knowledge of the etiology of the disease, the complexity of the biology and the predictive value of disease models. Also after drug approval by the Food and Drug Administration (FDA, USA) or European Medicines Agency (EMA, Europe), unexpected toxicity or lack of clinical benefit remains an important factor for drug withdrawal.[3] Therefore, in order to make better and safer drugs and to achieve higher success rates in the clinic, it is necessary that better drugs are pre-selected before these reach clinical development.

Physiological relevance of 2D in vitro disease models to predict drug efficacy

In order to pre-select better drugs for clinical development, it is necessary to have a more detailed look at the earlier stages of the R&D pipeline. After the identification of a druggable target for a disease, potential drug candidates that modulate the activity of the target can be developed, and can eventually be tested in a biological model.

When potential drug candidates are first investigated in cell-based biological models (*in vitro*), generally their efficacy is assessed on two-dimensional (2D) cell culture models, which are often named monolayers (figure 2). In such a cell culture system, cells that are relevant to the investigated disease or process are cultured on culture plastic in growth medium, supplemented with animal serum and often antibiotics. These cell models are generally easy to maintain and cheap to use, the latter of which is extremely important when testing many thousands of candidate molecules at once, often referred to as high-throughput screening (HTS). However, in recent years it has become increasingly clear that for many diseases, these monolayer cultures often fail to predict drug efficacy in animal models (*in vivo*) or in clinical trials.

A large problem associated with this poor translation is that many drug candidates that appear to be successful in such an *in vitro* model, fail in the later, more

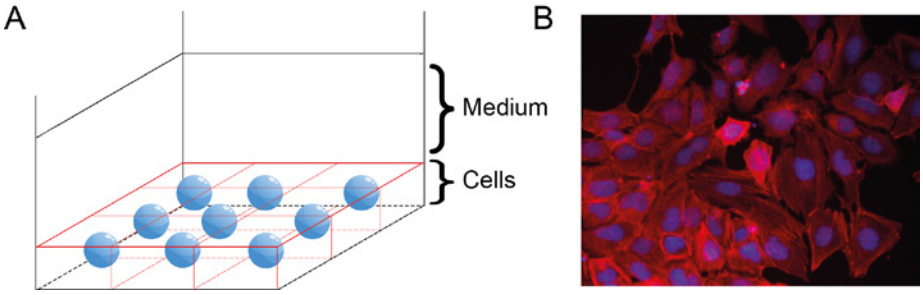

FIGURE 2 Cells cultured as a monolayer are poor simulators of human biology.
A) Schematic representation of cells cultured as a monolayer on culture plastic. Red lines represent the cortical cytoskeleton and nuclei are shown as blue spheres. **B)** Immunofluorescence image of 2D-cultured mIMCD3 cells (BD Pathway 855, 10x objective). Red colour shows cytoskeleton (rhodamine-phalloidin) and blue colour shows nuclei (Hoechst 33258).

expensive, drug development stages. Conversely, it is possible that potentially good drugs fail to show desirable effects in monolayer cell cultures, causing these to be filtered out and never progress into further development. A key concept in this problem is that monolayer cultures cannot adequately recapitulate the complex conditions in the body, since tissues are comprised of many cell types that interact within a three-dimensional environment. Hence, the tissue architecture that is observed in the body cannot be adequately recapitulated using monolayer cell culture models, since these fail to reflect the tissue architecture and its relevance in various disease processes. This, coupled to the implementation of the three R's (reduction, refinement, replacement) to reduce the use of animals in drug testing[5] means that more relevant *in vitro* evaluation models are required to select better drugs.

In order to provide a background for the different disease areas described throughout this dissertation, the following subchapters give a brief overview on two neoplastic disorders, cancer and polycystic kidney disease (PKD), where tissue architecture is essential for the pathophysiology and thereby providing a rationale for the development of new *in vitro* disease models on which to test candidate drugs.

CANCER

Disease background

Cancer is a neoplastic disorder that is characterized by abnormal cell proliferation and is among the most common causes of death worldwide.[6] The process by which healthy cells can transform into cancer cells and form a tumour is a multistep process by which cells need to acquire properties that confer a proliferative advantage, such as self-stimulatory growth signals, insensitivity to anti-growth signals, the ability to evade apoptosis and avoid immune destruction and the ability to induce angiogenesis. These

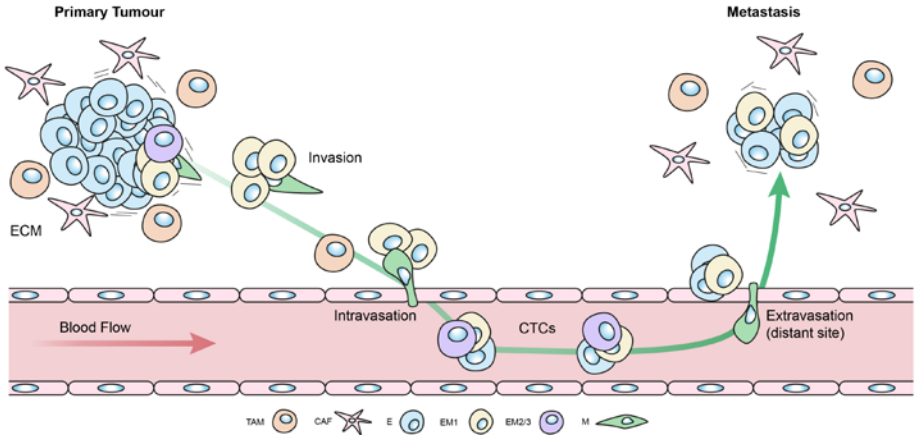

FIGURE 3 **Schematic representation of the metastatic cascade showing the processes of invasion, intravasation and extravasation at a distant site to establish a metastasis.** Extracellular matrix, ECM; Circulating tumour cells, CTCs; cancer-associated fibroblast, CAF; tumour-associated macrophage/M2-type macrophage, TAM. E (epithelial), EM1-3 (intermediate stages/partial EMT) and M (mesenchymal) represent different stages of EMT. Figure based on Nieto et al, 2016.[9]

factors, amongst many more, contribute to the origin and growth of a primary tumour[7-8] and are generally the result of acquired mutations that confer a growth advantage.

Importantly, the main cause of cancer-related deaths generally is not the presence of the primary tumour, but rather the metastasis of tumour cells to distant sites, where the growth of tumours can interfere with normal organ function. In order for metastasis to occur, tumour cells need to acquire migratory properties. These properties can then allow the cells to escape from the primary tumour into the blood stream (or lymphatic system), and eventually extravasate at a distant site. This metastatic cascade is illustrated in figure 3, and describes the process of invasion of tumour cells into the extracellular matrix, the intravasation into the blood stream and eventual extravasation at a distant site.[9] In general, the cause of this migratory phenotype is the loss of cell-cell contacts and changes in cell-matrix contacts and the secretion of matrix-remodelling enzymes. Collectively, this switch in cellular behaviour is often termed epithelial- to mesenchymal transition (EMT). It is currently becoming clearer that the tumour extracellular matrix[10-11] and many immune cells[12-15] play a role in carcinogenesis. Importantly, the causes of cancer are highly diverse, and range from genetic predisposition to DNA damage to diet.[16] Specific signalling pathways that are involved in this process are therefore highly variable and also depend on the tumour type and its underlying mutations. It is therefore not feasible and also not the scope of this chapter to discuss all these properties in detail, and the reader is referred to other relevant literature.[7-9, 16-18]

Current therapeutic strategies and limitations

Surgery is often the first line of treatment against a primary tumour that has not yet metastasized, sometimes supplemented with radiation- or chemotherapy, if required. Whether this strategy is successful depends highly on the tumour type, its underlying mutations and the tumour stage. For tumours that have already metastasized, surgery on its own is often not sufficient to cure the patient, and it is therefore often supplemented with radiation-, chemo- or immunotherapy. These additional therapies generally function to inhibit tumour cell proliferation, taking advantage of the tumours' defective DNA repair mechanisms, or to eradicate tumour cells by the immune system. However, such therapies, with the possible exception of immunotherapy, usually have side effects related to their effects on healthy cells. For example, the chemotherapeutic drug cisplatin (cis- diamminedichloroplatinum(II), CDDP) is a molecule that intercalates directly into the tumour cells' DNA,[19] thereby preventing cell division and tumour growth. However, the use of this molecule is limited by its nephrotoxic effects,[20] which are, at least in part, attributable to active cisplatin uptake in the kidneys by high affinity copper uptake protein 1 (Ctr1)[21] and organic cation transporter OCT2 (SLC22A2),[22] causing the local increase in intracellular cisplatin concentrations that is responsible for its nephrotoxic side effects. While molecules such as cisplatin can be effective at preventing the growth of tumours and their metastases, many such molecules do not effectively eradicate 100% of the tumour cells. Such drugs have mostly been developed using 2D-cultured, immortalized (and rapidly proliferating), tumour cell lines, most of which have retained little resemblance to the tumour they were originally derived from. Critically, because tumours are comprised of more than one cell type, some cell types are often unaffected by these proliferation-inhibiting drugs, which can in turn be responsible for tumour re-growth and therapy resistance.

Another strategy to improve patient survival is to prevent cancer metastasis, by blocking processes such as angiogenesis, cancer cell invasion into the surrounding matrix, intravasation or extravasation. Especially in this context, conventional 2D cell culture models represent a poor representation of the *in vivo* situation, since they lack the presence of extracellular matrix to model these processes. As one of the main topics of this thesis, we describe the development of a more physiologically relevant cell culture assay that can be used to study cancer cell invasion (the first step of the metastatic cascade) and to test treatments to prevent this process.

POLYCYSTIC KIDNEY DISEASE

Genetic background

Polycystic kidney disease (PKD) is a genetic disorder in which fluid-filled cysts develop in the kidneys (figure 4). In principal, these cysts develop in all segments of the nephron,[23] the kidney's smallest functional unit, but have been described to originate more often from the collecting duct.[24] As more and more cysts develop and grow over a patient's lifetime, kidney function deteriorates to end-stage renal disease (ESRD), the point where a patient requires kidney transplantation for survival. PKD exists as

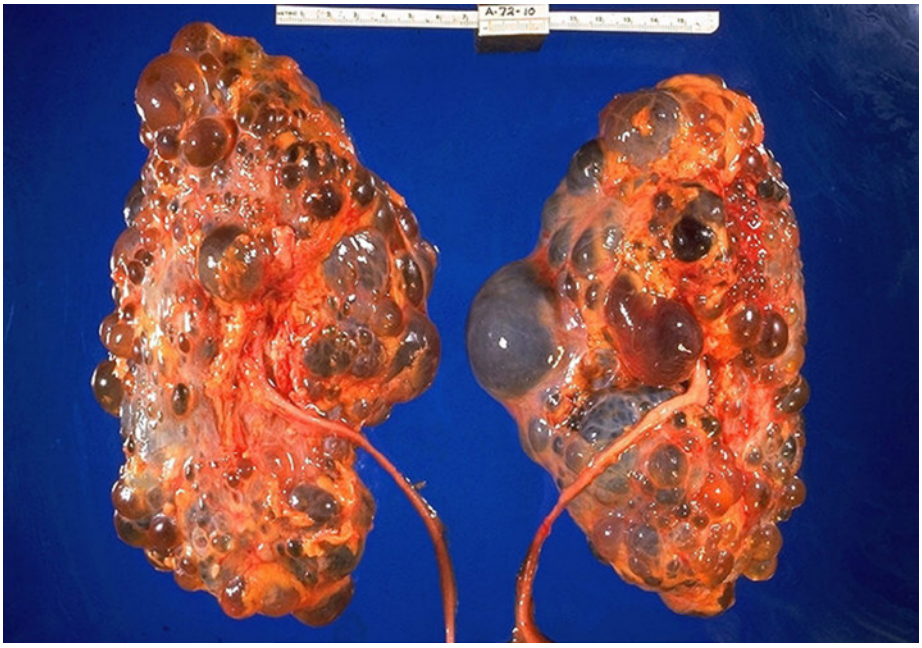

FIGURE 4 Pathology of polycystic kidney disease. Image provided by CDC/Dr. Edwin P. Ewing, Jr., 1972.

an autosomal dominant (ADPKD) and autosomal recessive (ARPKD) form. ADPKD is the most common form of the disease, which affects approximately 1 in 2500 people,[25] and thereby also places a large burden on society. The autosomal recessive form is much less prevalent (only approximately 1 in 20000 people[26]), but has a more severe nature. This specific form of PKD is often referred to as childhood PKD, because kidney function declines much faster than in the autosomal dominant form. Approximately half of the newborns that survive the neonatal period will develop ESRD within the first decade of life.[27]

In the case of ADPKD, a heterozygous genetic defect in either the *PKD1* gene on chromosome 16, or the *PKD2* gene on chromosome 4, underlies cyst formation, although the precise mechanism by which these mutations can cause the development of cysts remains largely unknown. One hypothesis that supports the slow progression of ADPKD is that heterozygous mutation of *PKD1* or *PKD2* is not sufficient to cause the formation of cysts, and inactivation of the second allele during life is required. This is supported by the finding that homozygous *Pkd1* or *Pkd2* inactivation in mice is embryonic lethal.[28] Additionally, renal injury may be an important contributor[29] to initiate cyst formation, as it is known that the presence of cysts can obstruct neighbouring tubules, likely leading to a cystic snowball effect that aggravates the cyst formation.[30]

PKD1 encodes the protein polycystin-1, which is a 467kDa transmembrane re-

ceptor-like molecule thought to be involved in mechanosensing[31] and cell-cell and cell-matrix interactions.[32] Moreover, this protein was recently identified as a receptor for various WNT ligands.[33] Polycystin-2 is a 110kDa polypeptide encoded by the *PKD2* gene, and this protein is known as a non-selective cation channel that is permeable to calcium ions.[34] Polycystin-2 is often named transient receptor potential polycystic 2 (TRPP2). ADPKD as a result from mutations in *PKD2* is generally milder, since cysts develop later.[35] The polycystin proteins can bind to each other[36] and form a functional complex[37-38] which is thought to be involved in the translation of mechanical stimuli to an influx of Ca^{2+} into the cell. This process is thought to be mediated by the localization of this complex to the tubular cells primary cilium, an organelle protruding from the cell membrane. However, the polycystin proteins localize also to different parts of the cell, such as the endoplasmic reticulum and cell-cell and cell-matrix contacts,[39-41] where they likely perform different functions ranging from mechanotransduction to regulating planar cell polarity (PCP).

For the autosomal recessive form of PKD, mutations in the *PKHD1* gene are responsible for the early onset and rapid progression of cystic kidney disease. It is estimated that such mutations are carried by approximately 1:70 people.[27, 42] The *PKHD1* gene encodes for the protein fibrocystin, also known as polyductin. Fibrocystin is a receptor-like protein for which ligands are currently unknown, and it can form a complex with polycystin-2.[43] Due to the lower prevalence of ARPKD, the following sections of this subchapter will focus instead on ADPKD. For more insight into the mechanistic background of ARPKD and its similarities with ADPKD, the reader is referred to other literature.[26]

Signalling alterations in ADPKD

Inactivating mutations in the genes responsible for ADPKD result in dysregulated cellular signalling pathways, with reduced intracellular Ca^{2+} levels as a central mediator. This reduction of Ca^{2+} can in turn activate calcium-inhibitable adenylyl cyclase (AC), to stimulate 3'-5'-cyclic adenosine monophosphate (cAMP) production. Conversely, reduced Ca^{2+} levels can also inhibit calcium-dependent phosphodiesterases (PDEs) to prevent cAMP breakdown. Alternatively, abnormal activation of the vasopressin V2 receptor (V2R) by antidiuretic hormone arginine vasopressin (AVP) can also drive the accumulation of cAMP through the activation of AC. This mechanism, together with the alterations in calcium homeostasis, has a central role in the pathophysiology of ADPKD.[44]

cAMP is an important second messenger, and the increased levels lead to many changes in cellular signalling, including increased activity of B-Raf, ERK, mTOR and PI3K pathways and of various proteins involved in the cell cycle and fluid transport (figure 5).[44-47] Together, these changes lead to increased cell proliferation, dedifferentiation and increased fluid secretion. These signalling alterations likely lead to the initiation of cyst formation and the consequent expansion that eventually causes renal failure.

Current therapeutic strategies

Currently, treatment for polycystic kidney disease is mainly aimed at alleviating fre-

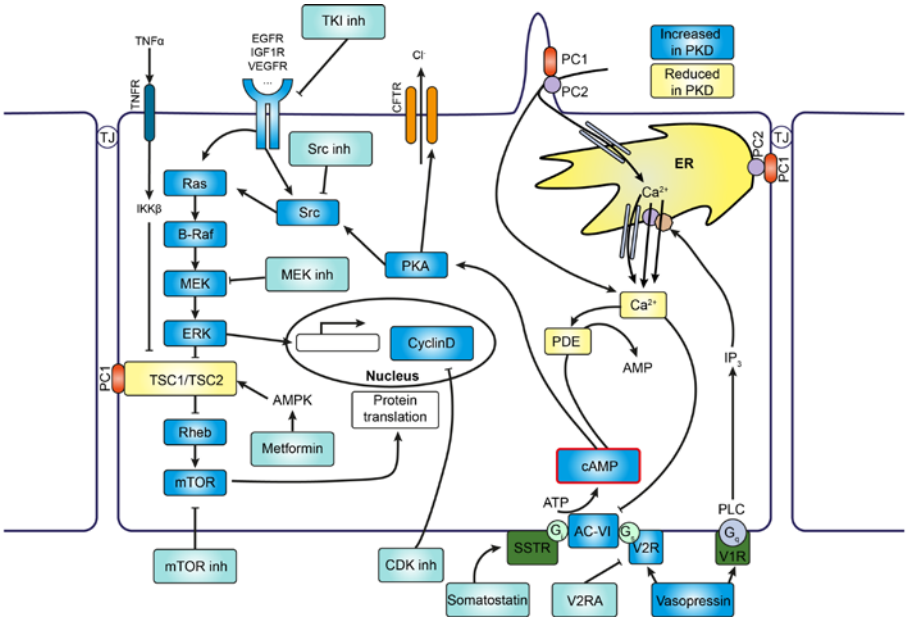

FIGURE 5 Schematic representation of known signalling alterations in ADPKD. Figure based on Torres et al, 2007 and Torres, 2010.[45, 110] Adenosine monophosphate (AMP), 5'AMP-activated protein kinase (AMPK), Adenylyl cyclase 6 (AC-VI), 3',5'-cyclic AMP (cAMP), cyclin-dependent kinase (CDK), cystic fibrosis transmembrane conductance regulator (CFTR), endoplasmic reticulum (ER), epidermal growth factor receptor (EGFR), extracellular regulated kinase (ERK), inositol triphosphate (IP_3), insulin-like growth factor 1 receptor (IGF-1R), mammalian target of rapamycin (mTOR), mitogen-activated protein-kinase/ERK kinase (MEK), phosphodiesterase (PDE), phospholipase c (PLC), polycystin 1 (PC1), polycystin 2 (PC2), protein kinase A (PKA), somatostatin receptor (SSTR), tuberin (TSC2), hamartin (TSC1), tyrosine kinase inhibitor (TKI), tumour necrosis factor alpha (TNFα), TNF receptor (TNFR), vascular endothelial growth factor receptor (VEGFR), vasopressin V1 receptor (V1R), vasopressin V2 receptor (V2R), V2R antagonist, (V2RA).

quent disease-associated symptoms such as hypertension,[48-50] cyst infection,[51] and pain.[45, 52-53] The patient will ultimately require renal transplantation, when the kidney function has deteriorated to the point of ESRD. However, as kidneys for transplantation are not always available, there have been many efforts to find a medicinal treatment to prevent disease progression. Due to the genetic background of the disease, it is amenable that genetic screening to allow early detection of the disease combined with pharmacological treatment that delays progression of the disease is sufficient to allow for a lifetime without disease symptoms. However, due to the extensive pathway deregulations in PKD (figure 5), the identification of effective drug treatments has been problematic.

Currently, the only therapy approved in the EU to slow disease progression is tolvaptan (marketed under the name *Jinarc*). This V2R inhibitor slowed down increases

in kidney volume and the decline in renal function in a recent Phase III clinical trial.[54] Blockade of the V2R prevents the binding of AVP to the receptor and prevents the consequent activation of AC, thereby delaying the growth of cysts. However, treatment with tolvaptan is also correlated with extensive side-effects that could limit patient compliance. These side-effects are largely related to the pharmacological action of tolvaptan and require patients to consume excessive amounts of water due to increased urine production. In addition, even though liver injury as a result of tolvaptan treatment is infrequent, patients receiving long-term tolvaptan treatment may be at risk of serious irreversible liver injury.[55-57] This illustrates that novel therapies are still needed.

In the past, there have been several clinical trials for mTOR inhibitors such as sirolimus (rapamycin) or everolimus. While mTOR inhibitors have often been proven effective in *in vivo* PKD models,[58-61] there have also been conflicting results.[62] In line with this, the clinical trials that have been performed for such inhibitors, have failed to show a clinical benefit.[63-68] However, it is possible that renal targeting of mTOR inhibitors like rapamycin can improve therapeutic response due to local increases in concentration.[69]

Other therapies currently undergoing clinical evaluation include somatostatin analogues (ALADIN trial), niacinamide, epidermal growth factor (EGF) inhibitors, and triptolide, (although a recent study with triptolide, NCT00801268, has been terminated due to high patient drop-out). A more detailed overview of the clinical trials undertaken for ADPKD has recently been published elsewhere.[70]

Limitations of 2D in vitro models

Even though 2D *in vitro* models for PKD have been useful to investigate signalling pathways, there are limits to their usefulness in evaluating the effects of potential therapeutics. Principally, the main pathophysiological characteristic, the growth of cysts, cannot be simulated in 2D, since a cyst is a three-dimensional structure. Very importantly, when test molecules are provided to 2D-cultured monolayers, the cells will be exposed to the test molecules on their *apical* side (figure 6A), whereas a closed cyst is more likely to take up a test molecule through its *basolateral* side (figure 6B). These differences in administration route could in turn lead to differences in intracellular concentrations, depending on transporter localization differences between *apical* and *basal* membranes.

Therefore, while the signalling pathways in PKD have often been investigated on 2D cell culture models, pharmacological treatment evaluation for PKD has traditionally been pursued in animal models, as treatment efficacy cannot adequately be measured using 2D cell culture. With the desire to reduce animal experimentation in mind,[5] new, more relevant, *in vitro* cell culture models for PKD need to be developed to facilitate preclinical testing of potential drugs. The development of relevant *in vitro* assays for PKD is therefore an important topic in this thesis.

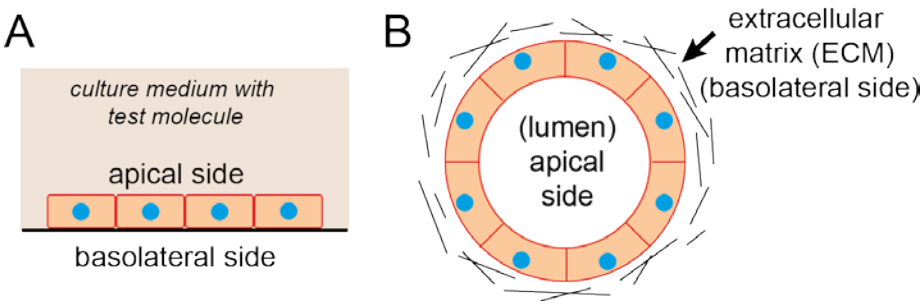

FIGURE 6 2D monolayers poorly recapitulate renal cysts. A) Schematic representation of cell polarity when cells are cultured as a monolayer. Apical side of cells is in contact with culture medium with test molecules. B) Schematic representation illustrating inverted orientation of cysts cultured in hydrogels. Basolateral side is in contact with the culture medium and test molecules.

3D cell culture models in drug discovery

In order to overcome problems traditionally associated with 2D cell cultures and to improve physiological relevance of *in vitro* cell models, the past couple of decades have witnessed the development of three-dimensional (3D) cell culture models. These models were developed in order to better model disease biology and bridge the gap between 2D *in vitro* models and the *in vivo* situation.[71] A great example that cells, when confined to a monolayer, display unnatural behaviour was proven when Bissell and colleagues showed that non-cancerous breast epithelial cells developed similarly to breast carcinoma cell lines when grown in 2D monolayers. However, when these cells were grown in reconstituted basement membrane (BM), non-cancerous cells responded to the presence of the BM by growth arrest, lumen formation and correct cell polarity, whereas the cancer cell lines were not growth-inhibited by the presence of BM.[72] Since then, several groups have proven large differences between 2D- and 3D-cultured cells, such as increased metabolic enzyme expression in liver cells which, may have profound consequences for *in vitro* toxicity assessment.[73-74] Additionally, the growth of tumour cells in 3D is known to enhance *in vivo*-like gene expression and structural properties.[75-78]

3D cell culture models in drug screening

Over the years, many different technologies have been optimized to allow compound screening on 3D cultured micro-tissues. Broadly, these techniques can be divided into scaffold-free and scaffold-based technologies, while the first category is largely comprised of multicellular tumour spheroids suspended in media, the second category comprises all matrix-embedded models such as the models used throughout this thesis.

Multicellular spheroids are clusters of cells that grow in suspension media. These media do not provide a rigid extracellular matrix and the cells are forced to aggregate

to a multicellular spheroid because cell-cell interactions dominate the cell-substrate interactions.[79-80] These cultures are known to be easy to prepare and provide physiologically relevant responses.[81] The cultures can be easily adapted in most labs, since there are many commercially available solutions, such as the hanging-drop microtiter plate (HDM) technology developed by InSphero AG. This technology can be readily scaled up to 96[82] and even 384 well plate formats.[83] Additionally, spheroids can be grown in the ultra-low attachment 96-well or 384-well plates commercially available from Corning as used here.[84] These 3D culture systems therefore have large potential in 3D high-throughput screening. Drawbacks of using such systems is that cells are required to produce their own extracellular matrix as they receive no support from the suspension media, and the limitation to the number of spheroids that are present in each well. Additionally, such a spheroid model may not recapitulate some aspects of tissue biology, since processes such as tubulogenesis, fibrosis and many more are not dependent on spheroid formation in the body. As an upside, the presence of suspension media rather than a gel makes it easier to collect 3D cultured cells for other techniques in molecular biology such as western blotting for the detection of proteins.

In contrast to the scaffold-free techniques, scaffold-embedded cell cultures are often used to prepare 3D cultures. These scaffolds are discussed further in more detail below, but mostly function to provide an extracellular matrix (ECM) to the cultured cells. Advantages of these scaffold-embedded cell cultures are that they are generally compatible with regular 96- and 384 well plates, and that the morphology of the cultured multicellular structures is not limited to the formation of spheroids. Additionally, the scaffold can often be modified to accommodate different cell types and behaviour, and multicellular structures can grow in all planes of the scaffold. While the increased number of structures in each scaffold can be considered as an advantage, it also poses new challenges for the analysis of results, as will be discussed in more detail in **chapter 2.** However, due to the presence of a scaffold, which is often rich in proteins, these 3D culture techniques are often not easily compatible with standard techniques such as western blotting, nucleic acid (RNA/DNA) extraction or immunofluorescent labelling.

ECM-mimicking scaffolds for 3D cell culturing

It is important to realise that the ECM that cells grow in are not 'passive', but highly contribute to cellular signalling. Consequently, a large effort was directed to the development of different ECM-mimics that simulate biological ECM properties.[85-88] Cell behaviour in 3D cell systems can be influenced by different matrix types. Importantly, the choice of matrix will depend on the cell type and aim of the study. In order to provide 3D cultured cells with more physiologically relevant microenvironments, hydrogels comprise a highly convenient and highly popular material for 3D culturing. However, many different types of hydrogel exist, and these gels can either be purely natural or synthetic.[85, 88] Natural hydrogels are derived from natural sources and thereby inherently support cell viability and promote cellular signalling. As a disadvantage of this type of natural gel, it is often impossible to completely define the matrix and due to their nat-

ural background, they can also suffer from batch-to-batch variability, which can induce biological variation in cultured cells.[85] The most popular types of natural gels include agarose, collagen (often collagen type I derived from rat tail tendons), fibronectin, laminin, silk fibroin, fibrin and matrigel,[89] the latter containing natural ECM components and proteins.[90] An advantage of these natural materials is that by altering the concentration it is possible to alter the gel rigidity or pore size, making it easy to modify the gel properties, which in turn alters cell behaviour.[91] Synthetic hydrogels, in contrast, are comprised of non-natural molecules, which have the advantage that the chemical composition of the resulting gels is highly reproducible and very well-defined.[85] An example of a synthetic hydrogel is poly-ethylene glycol (PEG), which can support cell viability.[85, 92-95] These gels, however, do not always have desirable biological properties and generally lack important factors to support cell viability and *in vivo*-like cell growth. In order to improve their biocompatibility, synthetic hydrogels may be readily altered to incorporate, for example, relevant integrin-binding domains by incorporating ECM proteins or relevant peptide sequences. In addition, it is possible to generate 3D matrices using a combination of natural and synthetic polymers, and these can be designed to resemble the natural ECM. For a more detailed review on the different synthetic and natural hydrogels and their properties, the reader is referred elsewhere .[85-86, 90]

Towards more relevant screening assays

Even though the currently available 3D culturing techniques have greatly improved physiological relevance of *in vitro* models, these models still have to be incorporated for routine drug screening.[96] Currently, the most common use of 3D cell culture assays is to validate observations made in 2D cultured cells. This can range from the validation of certain signalling cascades to validating selected hits obtained from compound screens. However, when validating hits from compound screens, it is possible that potentially good molecules have been filtered out due to screening in 2D cell cultures. It is therefore important to use more physiologically relevant assays in (primary) compound screening.

The main drawbacks for the incorporation of 3D cell culture assays in screening have traditionally been that such assays are often much more expensive than their 2D counterparts, making 3D assays unsuitable for large-scale compound screening. Additionally, 3D assays can encompass more biological variation, sometimes making it harder to obtain meaningful measurements. A solution to these problems has been provided by the adaptation of robotics for cell culture automation, and also the large-scale production of scaffolds in which to culture cells. Aside from these technological drawbacks, a case can be made against the use of transformed or immortalized cell lines in 3D assays. These immortalized cell lines are often used, also in this thesis, because they are relatively cheap to use and have little variation between batches. While these cells are therefore convenient for screening, it is important to mention that tissues are not composed of a single cell type, and more physiological characteristics may be captured by using a different cell model. Additionally, immortalized cell lines have often been passaged many times, which can change their (epi-)genetic and physiological

characteristics.[97-98] Even though, in 3D cultures, these cell lines may display more *in vivo*-like behaviour, the relevance to a functional organ may be improved by the incorporation of multiple (non-immortalized) cell types. On the other hand, for screening large molecule libraries, this addition of multiple cell types may induce undesirable variation into the model, making efficacy readouts less consistent.

In recent years, there also has been an increased interest in the use of tissue-derived stem cells and induced pluripotent stem cells (iPSC) for drug screening. Indeed, such cell types can be used to more accurately simulate organ function,[99-101] but often also require extensive differentiation procedures, which means that they can be more expensive to work with. Additionally, these cells often require fresh patient-tissue supplies, meaning that they are not always readily available for screening and may also vary more between different sources. However, such stem cells do open the door for more personalized medicine, and it is possible that, eventually, these cell types will become more popular than cultured cell lines.

Alongside the selection of the correct cell type, it is important to also select the correct matrix to culture cells in, so that they can organize in a functional way to form tissue-like structures. An interesting recent development has been the use of de-cellularized organs, where the cells have been removed and the extracellular matrix can be solubilized to form hydrogels. This approach has been applied for multiple organs,[102] such as colon,[103] heart,[104] kidney,[105] liver,[106] lung[107] and skin.[108] In its current status however, it is not likely that this can be applied in routine screening purposes. Another use for these de-cellularized organs can be found in organ replacement therapy, since de-cellularized organs can potentially be re-seeded with a patient's own cells to again form functional organs.[109]

Aim and outline of this thesis

In **chapter 2**, we discuss, in addition to the importance of screening in biologically relevant *in vitro* models, the importance of having a biologically relevant assay readout. As many groups that make use of 3D cultured cells still use relatively simple measurements to assess compound efficacy, such as biochemical toxicity measurements, or single phenotypic parameter readouts of 3D cultures, the complexity that is provided inherently by 3D cultures can be exploited by the application of phenotypic profiling of drug effects.

In **chapter 3**, we use a 3D cultured prostate cancer cell line to model tumour cell invasion as a result of growth factor stimulation. As mentioned earlier in this chapter, cancer cell invasion is one of the first steps that eventually leads to cancer metastasis. This 3D culture model was scaled up for use with 384 well plates and automated liquid handlers, so that many compounds could be screened simultaneously. This screening platform was applied to measure the activity and selectivity of inhibitors of the c-Met and epidermal growth factor (EGF) receptor (EGFR) tyrosine kinases. We could identify selective inhibitors of both c-Met and EGFR, and could also identify dual kinase

inhibitors. These findings were subsequently confirmed after *in vitro* enzyme activity measurements.

In **chapter 4**, we describe the development of a 3D cell culture-based high-throughput screening platform for PKD. This screening platform was applied to screen a tyrosine kinase inhibitor library of 273 small molecules with pre-described targets, to identify new druggable targets for PKD. Using multiparametric phenotypic classification of compound effects, we could discriminate desirable compound effects from potentially toxic molecules. Using this strategy, we identified many molecules that targeted kinases that are known to be involved in PKD, such as mTOR, CDK and Chk, but we failed to identify PI3K inhibitors as effective molecules, even though this pathway is known to be dysregulated in PKD. Additionally, we found targets that have not been previously described in PKD, such as Syk. In this chapter, we show that using *in vitro* models with high pathophysiological relevance coupled to phenotypic profiling can be used to predict and validate molecular targets.

In **chapter 5**, we further applied the methodology developed in **chapter 4** to screen a kinase inhibitor library to investigate pathways involved in cystogenesis. We discovered that most active molecules overlapped in target specificity with **chapter 4.** However, in order to discriminate potentially non-specific molecules, we screened the entire molecule library using a tumour cell invasion model. This strategy allowed us to prioritize molecules that affected cystogenesis but not tumour cell phenotype.

In **chapter 6**, we applied the 3D cell culture model developed in **chapter 4** to screen a SPECTRUM compound library containing over 2320 molecules to find potentially new therapeutics against PKD. We found that 81 of the 2320 molecules potently inhibited cyst growth, and using multiparametric phenotypic measurements we excluded potentially cytotoxic molecules. We selected two molecules, pyrvinium pamoate, an antihelmintic drug, and celastrol, a triterpenoid derived from *Tripterygium Wilfordii*, for *in vivo* evaluation in an iKspCre-*Pkd1*^{lox,lox} mouse model of PKD. In contradiction with the effects observed *in vitro,* we did not observe beneficial effects of pyrvinium pamoate on kidney volume and function. However, we discovered that cyst growth was markedly reduced after treatment with celastrol. In addition, celastrol prevented the associated decline in renal function and also ameliorated tissue fibrosis that normally accompanies cyst growth.

Chapter 7 provides a general discussion to discuss the conclusions in this work and the implications of the work presented in this thesis.

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