evotec

DRUG DISCOVERY UPDATES #12

DDup

EVOTEC'S 3D CELL MODEL PLATFORM

Predict drug efficacy and toxicity by using next generation *in vitro* cell systems

INTRODUCTION TO 3D CELLULAR MODELS TO ADVANCE DRUG DISCOVERY

3D *IN VITRO* PLATFORMS

INTERVIEW WITH *Reiner Class & Christodoulos Xinaris*

5 QUESTIONS TO *Magali Ferro & Stephanie Ryder*

2022/03

evotec — DDup

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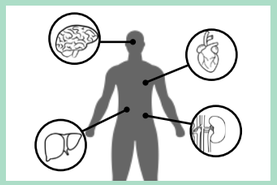

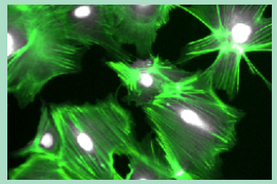

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evotec

DEAR FRIENDS
OF EVOTEC

A message from Evotec CEO
Dr Werner Lanthaler

Welcome to this twelfth issue of DDup, an Evotec publication providing you with more insights into the company and its capabilities. In this DDup edition, we would like to introduce our exciting new developments in pre-clinical cellular models with a particular focus on three-dimensional (3D) cell culture platforms, including microtissues, organoids and organ-on-a-chip systems. Improving the accuracy of efficacy and toxicity prediction of drug candidates in the future will lead to the development of high quality novel therapeutics with an enhanced safety profile.

Prescribed 3D *in vitro* platforms will be an important element of Evotec's R&D Autobahn and will, in early and late drug discovery, provide advanced knowledge in terms of efficacy and toxicity. This can drastically improve our prediction of potential *in vivo* issues meaning that well-informed decisions can be made much earlier than it is possible today, thus saving time, effort and cost. The development of these platforms is another great example of a cross-site effort, with dedicated scientists in Germany and the UK working closely together, showing again that Evotec with its global presence is acting as ONE team. This exciting development work is one more example of what has been established at Evotec in the very recent past, beside others, our comprehensive panOmics platform, a next generation predictive toxicology platform, or PanHunter, Evotec's next generation multi-omics data analysis platform, cementing Evotec's position as one of the most innovative players in the drug discovery space.

Thank you for reading this latest edition of DDup – we hope you found it of interest. We welcome your thoughts and input, and hopefully we will get the opportunity to collaborate in this exciting area of science in the future.

Yours sincerely,
for the management of Evotec
Werner Lanthaler,
CEO of Evotec SE

CHAPTER 01

INTRODUCTION

TO 3D CELLULAR MODELS TO ADVANCE DRUG DISCOVERY

In the drug discovery process, pre-clinical testing serves as a foundation for predicting efficacy and evaluating the potential toxicity of drug candidates in humans. With the advent of state-of-the-art technologies such as tissue engineering and microfabrication, we have the opportunity to build complex *in vitro* cellular models that recapitulate the human physiology of tissues and organs in a dish. In this DDup edition, we would like to introduce our exciting new developments in pre-clinical cellular models with a particular focus on three-dimensional (3D) cell culture platforms, including microtissues, organoids and organ-on-a-chip systems. These models serve as a tool to improve our understanding of human diseases, support the identification of novel therapeutics and better predict the human response to drug candidates.

THE LACK OF RESEARCH TRANSLATABILITY

Moving a drug from bench to bedside involves extensive characterisation for safety and efficacy in pre-clinical studies using human-based cellular systems and animal models. The drug development campaign is lengthy (up to 15 years) and costly (up to $ 2.6 billion), with a high attrition rate; on average only one drug out of 1,000 tested gains FDA approval. The main reasons for drug programme terminations are the lack of drug efficacy and safety at clinical trial phases. The lack of translational relevance from pre-clinical models to human scenarios is a primary cause of the failure. The inadequate representation of the human tissue environment in pre-clinical models may result in poorly validated therapeutic targets and inaccurate predictions of the drug candidate's effects on humans.

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THE CHALLENGE OF KIDNEY DISEASE MODELLING

Acute kidney injury (AKI) and chronic kidney disease (CKD) are disorders negatively impacting on kidney functions and structure, affecting about 10% of the population worldwide. Animal models are widely used to study human kidney disease induction and progression using endpoints such as:

- Urine volume
- Urine albumin level
- Serum creatinine level
- Glomerular filtration rate (GFR)

However, as for many disease areas, animal studies present significant drawbacks with inherited species-specific differences, this is particularly true in kidney diseases. To date, none of the pre-clinically validated drug candidates for AKI have been confirmed in clinical studies, mainly due to the lack of efficacy in patients. At the same time, standard human cellular models with one cell type cultured on a plastic substrate do not accurately depict and simulate the structure and functional characteristics of the human kidney. Therefore, the pharmaceutical industry has pushed toward developing more predictive models of the human kidney by including vascular perfusion, cellular crosstalk and mechanical forces which helps recapitulate *in vivo*-like features and functions.

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THE CHALLENGE OF DRUG-INDUCED TOXICITY PREDICTION

During pre-clinical stages, the safety profile of drug candidates is established using *in vitro* cellular and *in vivo* animal models and aims to identify and characterise the biological mechanisms responsible for organ toxicities. Nonetheless, adverse drug events such as cardiotoxicity, hepatotoxicity, and other organ toxicities account for 30% of drug failures during drug development programs. Most of these safety liabilities are identified during late-stage clinical development or post-marketing, wasting valuable resources and risking lives. Ultimately, this observation suggests traditional simplistic 2D *in vitro* models poorly recapitulate the healthy or diseased tissue resulting in poor translational relevance to the patient. Given the complexity of human biology and the increasing variety of drug modalities, pre-clinical strategies have evolved over recent years to incorporate more sophisticated human-relevant *in vitro* cell-based models that integrate the structural and communicative complexity of tissues whilst permitting multiparametric endpoint analysis. The overall aim being refinement of our risk assessment predictions.

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IMPROVING HUMAN DISEASE MODELLING USING 3D CELLULAR MODELS

It is now recognised that the traditional approach to establishing pre-clinical cellular models, often utilising cancer cell line-derived monolayers within 2D plastic plates, has its limitation in the prediction of human biology. For example, to model solid organs that exhibit complex compartmentalised microstructures, such as the liver and the kidney, using simple 2D models is not possible. Over the past 10 years, much interest has been placed in establishing cellular models that mimic the tissue and organ microarchitectures better by allowing multi-cellular interactions in a 3D structure. Significant technological advances in tissue engineering and microfabrication supported the development of numerous 3D cellular models, from simple spheroids or microtissues to more complicated iPSC-derived organoids and microphysiological systems. The integration of mechanical factors such as stretch or perfusion (e.g., blood flow) emulates the fluid tissue-level functionality. It integrates the cellular response to mechanical stress, sometimes a direct cause of organ injury. The development and validation of these human-relevant complex cellular models for drug discovery programs could improve pre-clinical findings and clinical outcomes.

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THE PROMISE OF 3D CELLULAR MODELS IN PRE-CLINICAL SAFETY TESTING OF NEW DRUGS

Efficacious drugs can cause unwanted toxicity effects; the leading causes of drug attrition within the clinic are drug-induced liver and cardiac toxicity. These adverse drug reactions (ADR) may arise from direct interactions with cellular components of a target tissue or indirect interference within the closely regulated cellular communications and thus functionality of the tissue structure. As a result, using 2D single-cell monolayer models for drug safety testing makes accurate predictions of drug toxicities challenging. 3D spheroid models have emerged as a promising *in vitro* tool to enhance *in vitro* to *in vivo* translatability while maintaining the high-throughput requirements of early pre-clinical drug screening. Spheroids are cost-effective compared to other 3D cellular models, such as iPSC-derived organoids, while allowing the culture of single or multiple cell types as aggregates exhibiting high cell-cell and cell-matrix interactions. The resulting spheroidal micro-tissues demonstrate enhanced biological activity and increased longevity in culture, thus bridging the *in vivo* gap by allowing repeat dosing strategies to be assessed *in vitro.*

	iPSC-derived cells	Spheroids/ Microtissues	Organoids	Microphysiologic system
Model description	Cells derived from iPSCs under fully defined induction condition	3D cultured iPSC-derived cells or primary cells as a single or mix of multiple cell types	Organotypic tissue from iPSC-derived cells, self-organised into mini organs	Engineered organ substructures made from iPSC-derived cells or primary cells cultured in a microfluidic device
Target organ(s) at Evotec	Cardiac / brain / kidney	Cardiac / brain / liver	Kidney	Kidney
Area of use at Evotec	Pre-clinical safety / disease modelling	Pre-clinical safety	Disease modelling	Disease modelling

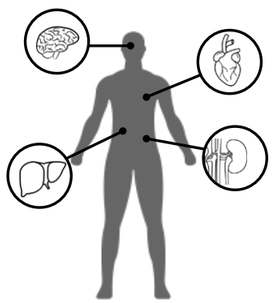

Figure 1: Definition of 3D in vitro cellular models and description of their area of use at Evotec

CHAPTER 02

EVOTEC'S 3D *IN VITRO* PLATFORMS

In this second section of this DDup, you will discover how Evotec has concentrated its effort in building complex cellular models and integrating them into the drug discovery pipeline. These human-relevant models recapitulate key physiological features of the tissue in healthy and disease states and offer the opportunity to predict clinical outcomes better. You will first learn about Evotec's kidney-on-a-chip and organoid models to study the induction and progression of human kidney disease and to discover new therapeutic targets. You will then learn about Evotec's portfolio of microtissue models covering liver, heart and brain and how these models are utilised within high-content and high-throughput *in vitro* toxicology studies to improve lab to human translation as early as compound library screening.

GLOMERULUS-ON-A-CHIP TO STUDY KIDNEY DISEASE

The nephron is the functional unit of the kidney. It comprises two important parts: the glomerulus, responsible for serum hyperfiltration, and the proximal tubule, which plays a key role in the reabsorption of water, ions and other solutes. The glomerular filtering function is linked to its tri-layered tissue structure composed of a basement membrane separating podocytes and glomerular endothelial cell layers. Glomerular diseases (GDs) affect the glomerular filtration barrier (GFB) through various pathways and across both cell types. GDs are involved in 70% of diagnosed kidney diseases and multiple systemic diseases, such as diabetic nephropathy. Existing treatments focus on the underlying cause of the GDs, when this is known, to protect the kidney from further damage. However, no therapeutic options are targeting the glomerulus directly.

Suitable *in vitro* cell models to study GDs are challenging to establish. On the one hand, they require access to human podocytes that are comparable to human cells *in vivo* and, on the other hand, these podocytes need to be cultured in a system that mimics the dynamic microenvironment associated with blood filtration. Immortalised human podocyte cell lines often lack morphologically and functionally relevant features of their *in vivo* counterpart. Access to primary podocytes is limited and the invasive sourcing of human podocytes, as well as prolonged *in vitro* culture pose a risk of losing the expression of key functional and structural proteins. To overcome this limitation, Evotec, together with the Mario Negri Institute Bergamo, Italy, has developed a protocol to produce efficiently and robustly industry scale, high-quality human-induced pluripotent stem cell (iPSC)-derived podocytes. The main advantage of using iPSC technology is the access to patient iPSCs, allowing the study of disease phenotypes and mechanisms within the context of the patient genome. These iPSC-podocytes contain an extensive actin cytoskeleton and express essential physiological proteins, such as nephrin and podocin, necessary to form a selective filtration barrier *in vivo*. In many GDs, podocytes undergo actin cytoskeletal remodelling or detachment from the glomerular basement membrane. These cellular and tissue defects may serve as a potential target to develop new drugs.

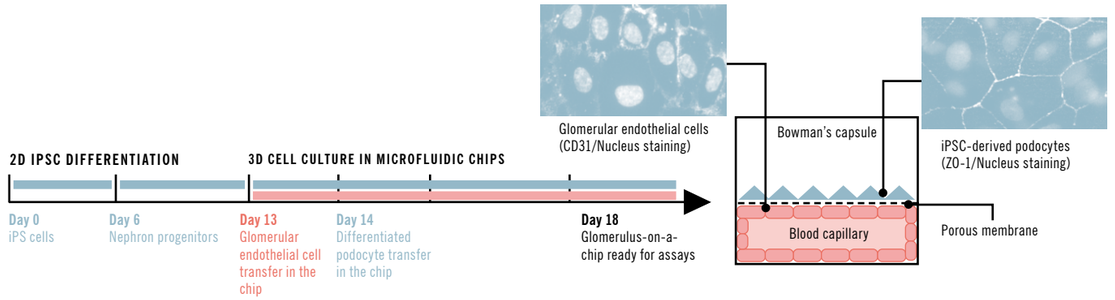

Figure 2: iPSC to glomerulus-on-a-chip: steps and time course. *iPSC-derived podocytes and primary glomerular endothelial cells are cultured in 2D separately to allow for maturation in their optimal medium before being co-cultured into the microfluidic chip for a minimum of 4 days to form a glomerular filtration barrier.*

The kidney's GFR is the best index of kidney function. The GFR is calculated as the ratio of urine albumin to serum creatinine and is classified into five categories staging the kidney injury level. Most GDs are associated with a decrease in GFR and a subsequent increase of albumin in the urine (also called albuminuria). To study the induction and progression of GDs, Evotec has built a glomerulus-on-a-chip platform by culturing iPSC-podocytes in a microfluidic device, allowing podocytes to experience physiological flow associated with blood filtration. Using this platform, we have established a functional assay to quantify the natural ability to selectively filter small waste molecules and retain proteins such as albumin of the iPSC-podocyte layer. By perfusing various chemical stimuli within our podocyte-chip, we successfully modelled a range of kidney injuries, including diabetic nephropathy, nephrotic syndrome (the most common CKD in children) and drug-induced nephrotoxicity. We were able to show the utility of the glomerulus-on-a-chip platform by analysing the impact of these chemical stressors on iPSC-podocyte cellular features and tissue functionality. Using various phenotypic and functional assays, we showed impairment in podocyte structure with significant actin remodelling, cell detachment from the glomerular basement membrane, and hypertrophy. These disease phenotypes result in loss of filtration barrier integrity as measured by an increase in albumin permeability. This mechanistic assay allows us to have comparable endpoints between pre-clinical cellular models, animal models and clinical studies and enhance data translation. The glomerulus-on-a-chip platform enables us to perform longitudinal studies of GDs and understand the disease evolution better, thereby increasing our chances of identifying and validating new therapeutic targets and developing meaningful drugs that benefit patients.

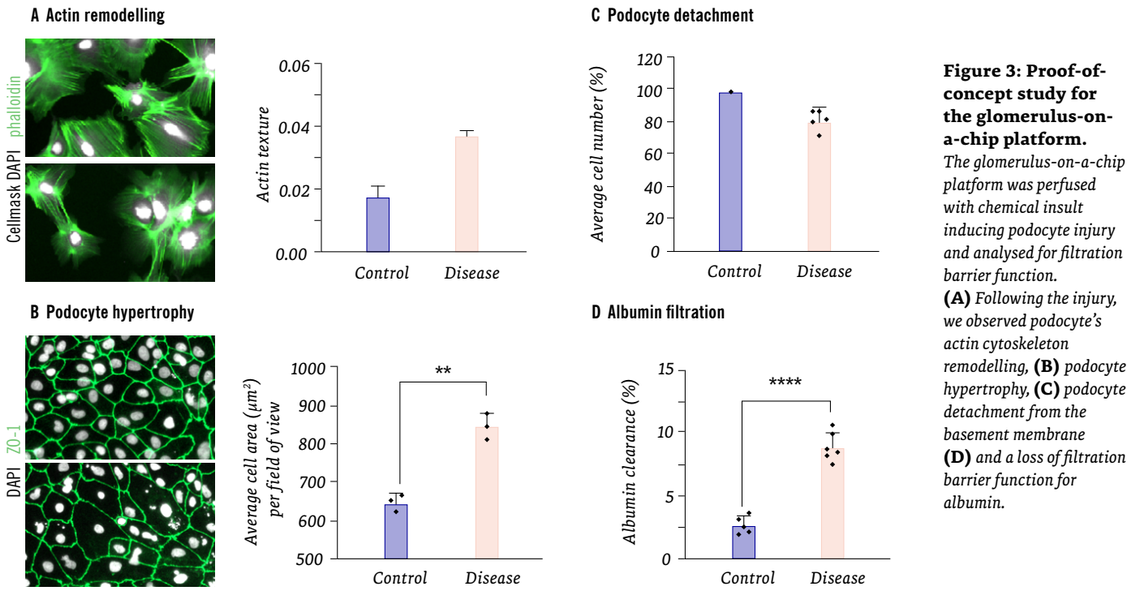

Figure 3: Proof-of-concept study for the glomerulus-on-a-chip platform. *The glomerulus-on-a-chip platform was perfused with chemical insult inducing podocyte injury and analysed for filtration barrier function.* **(A)** *Following the injury, we observed podocyte's actin cytoskeleton remodelling,* **(B)** *podocyte hypertrophy,* **(C)** *podocyte detachment from the basement membrane* **(D)** *and a loss of filtration barrier function for albumin.*

To reproduce the intricacies of the human glomerulus, including the communication between cell types, Evotec is also building a complex glomerulus-on-a-chip model that integrates primary human glomerular endothelial cells at the interface with iPSC-podocytes, thus reproducing the tri-layered structure of the tissue.

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KIDNEY ORGANOIDS TO STUDY POLYCYSTIC KIDNEY DISEASE

Autosomal dominant polycystic kidney disease (ADPKD) is the most common form of polycystic kidney disease (PKD). Patients present with fluid-filled cysts, swelling and functional decline of the kidneys, ultimately leading to end-stage renal disease (ESRD). With a prevalence of between 1/400 and 1/1,000 live births, this monogenic disease affects more than 10 million people globally. Even though mutations in PKD1 or PKD2 (encoding for PC1 and PC2) have been identified as responsible for most APDKD cases, PC1/PC2 interaction and underlying mechanisms driving disease progression are poorly understood. To validate new therapeutic targets and to test compounds in pre-clinical development, human-relevant *in vitro* systems are indispensable. Traditional *in vitro* systems to model ADPKD are limited to immortalised cell lines cultured in 2D or donor tissue, lacking good predictivity or donor-to-donor variability. iPSC-derived kidney organoids, therefore, offer an advanced *in vitro* system due to their multicellular nature and high physiological relevance. Through the iPSC differentiation process, disease relevant kidney cell types emerge in 3D in close proximity to each other, closely emulating the *in vivo* situation. Since ADPKD is such a complex disease, possibly due to the involvement of multiple kidney cell types, *in vitro* models must recapitulate this intricate system to enable the development of therapies. In addition, Evotec is currently developing a CRISPR CAS9 engineered ADPKD *in vitro* model in synergy with multiple teams from various sites. Derived from a cryopreserved iPSC PKD1-/- nephron progenitor cell population, kidney organoids will undergo cystogenesis evaluation in multiple drug efficacy read-outs with the perspective of supporting compound screening soon.

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ORGAN MICROTISSUES TO PREDICT DRUG-INDUCED TOXICITY

Liver is the main organ of clearance for many drugs due to its high metabolic enzyme capacity and biliary excretion function. Drug-induced liver injury (DILI) is a leading cause of drug withdrawal from the market; approximately 18% of compound withdrawals between 1953 and 2013 were due to hepatotoxicity events. Large-scale comparisons of *in vivo* animal to human toxicity events concluded only approximately 50% correlation, highlighting the clear need for human relevant models. To better predict DILI, we established a human liver microtissue (hLiMT) by co-culturing primary human hepatocytes with non-parenchymal cells in round bottom ultra-low attachment plates compatible with multiparametric high-content imaging and biochemical cytotoxicity assays. HLiMT's are functional as depicted by their metabolic activity (cytochrome P450), albumin production, bile canaliculi formation and long-term stability (up to 28 days). hLiMTs were exposed to a reference set of 54 compounds all of known DILI risk (29 high risk, 9 low risk and 16 with no risk) in a chronic repeat exposure period of 14 days. Utilising this model within our high-content multi-parametric screening methods we were able to predict the known DILI risks with an assay accuracy of 91%. To place this into context, our more traditional 2D human hepatocyte monolayer assay only achieves 70% accuracy.

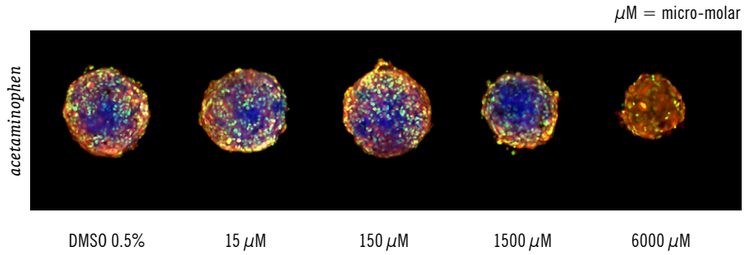

Figure 4: Representative high-content images of human liver MTs. *Representative 3D confocal high-content screening (HCS) images of human liver MTs exposed to known hepatotoxins, troglitazone and acetaminophen, labelled with Syto11 (green) to detect DNA structure, monochlorobimane (mBCL) (blue) to detect GSH content, dihydroethidium (DHE) (yellow) to detect ROS formation and MitoTracker deep red (red) to detect mitochondrial function.*

Drug-induced cardiovascular toxicity can result from functional (arrhythmias, reduced contractility) and structural (morphological) alterations to or within the myocardium. These effects can occur by directly target cardiomyocytes or indirectly by targeting the 70% non-myocyte cell population. To date human cardiovascular toxicity has been poorly detected in *in vitro* pre-clinical models due to a lack of model complexity or human relevance, with cancer-derived or primary animal cell types being utilised often in low output, low throughput analysis platforms. In recent years the advent of iPSC-derived cardiomyocytes capable of spontaneous beating has propelled our possibilities for *in vitro* cardiac strategies. To better predict drug-induced cardiotoxicity Evotec has utilised these human iPSC-derived cardiomyocytes within a spheroidal cardiac microtissue (CMT) by co-culturing with primary human cardiac microvascular endothelial cells and cardiac fibroblasts. CMTs display uniform size and shape (200-µm diameter) and the expression of key cellular markers representative of the differentiated cell types (CD31, vimentin & ACTN2). Spontaneous tissue contraction is visible after 5 days of culture and maintained for at least 28 days, which suggests similar function as the human myocardium. A comparison study was performed cross comparing the responses of CMTs with more traditional models; single cell monolayers of h9c2 or iPSC-cardiomyocyte.

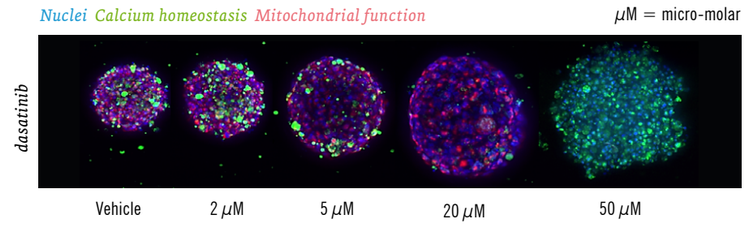

Figure 5: Representative high-content images of human cardiac MTs. Representative 3D confocal high-content screening (HCS) images of human cardiac MTs exposed to known cardiotoxin, dasatinib, labelled with Hoechst (blue) to detect DNA structure, fluo-4 AM (green) to detect calcium homeostasis, TMRE (red) to detect mitochondrial function.

All models were treated with 11 reference compounds (9 known cardiotoxins and 2 non-cardiotoxins) and morphological changes assessed using multi-parametric high-content imaging looking at known key mechanism of structural cardiotoxicity (mitochondrial and calcium destabilisation). CMT predicted the known human cardiotoxicity with 100% accuracy while h9c2 and iPSC-cardiomyocytes both achieved 81%.

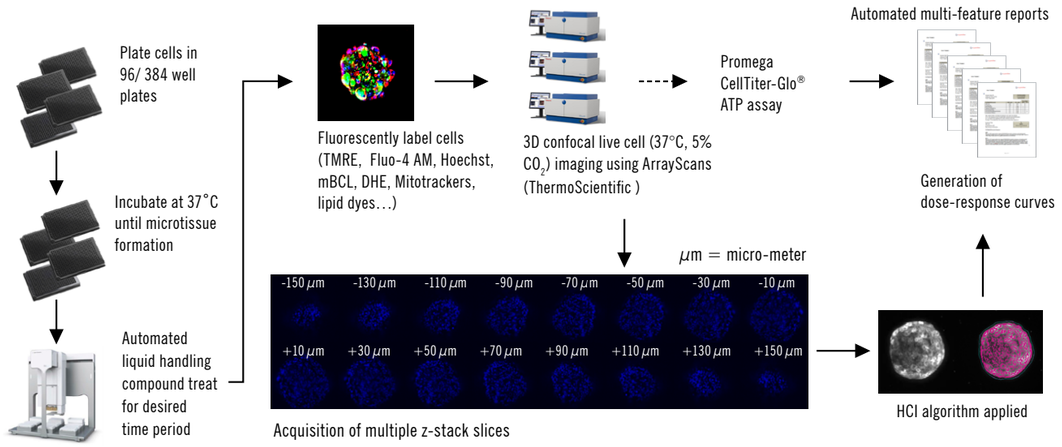

Figure 6: 3D high-content screening overview

The prevalence of adverse neurotoxic reactions of the brain in response to drugs or environmental hazards continues to prompt the development of novel cell-based assays for accurate neurotoxicity prediction. In response to this demand, Evotec built a mature iPSC-derived brain microtissue (BMTs) model comprising a mixed cell population of iPSC-neurons and iPSC-astrocytes. As with the hLiMTs and CMTs, the BMTs also display uniform size, shape and expression cell specific biomarkers (βII-tubulin and GFAP). The iPSC-derived astrocytes migrate over time in culture (28-day period) to form a clear coverage of astrocyte bodies around the periphery with a network of processes spanning the internal structure of the microtissue much like the basic cellular organisation of brain tissue. We used 10 reference compounds (8 known neurotoxins and 2 non-toxins) to validate the BMTs for neurotoxicity prediction looking at the effects of both acute and chronic compound exposure.

BMTs were analysed using multi-parametric high-content imaging of calcium homeostasis and mitochondrial function alongside intracellular ATP assessment. Interestingly we found no difference in toxicity prediction between acute and chronic compound exposure until we factored in brain tissue specific maximum therapeutic concentration levels rather than the more routinely utilised total blood plasma concentration levels. Using this technique our assay accuracy jumped from 60% to 80% with chronic compound exposure only. Firstly, highlighting the importance for chronic compound treatment within *in vitro* strategies but also how critical it is that we interpret the data correctly. When attempting to refine our *in vitro* prediction of brain neurotoxicity it comes as no surprise that the critical blood-brain barrier must be factored in when we analyse our *in vitro* findings.

		Toxicity Prediction		
Microtissue Model	**Endpoints**	**Sensitivity**	**Specificity**	**Accuracy**
Liver	Size, GSH, ROS, MMP, MitoMass, ATP	91%	100%	91%
Cardiac	Size, calcium, MMP, MitoMass, ATP	100%	100%	100%
Brain	Size, calcium, MMP, MitoMass, ATP	88%	50%	80%

Figure 7: Predictive performance of 3D models relative to in vivo findings

5 MINUTES WITH

CHAPTER 03

DR REINER CLASS AND DR CHRISTODOULOS XINARIS

Dr Christodoulos Xinaris

Head of the Laboratory of Organ Regeneration at Istituto Mario Negri

Christodoulos studied Biology at the University of Athens and received his PhD for work done in the field of Cell and Molecular Biology at the Athens Medical School. In 2008, he joined the Mario Negri Institute for Pharmacological Research first as a Marie Curie Post-doctoral Fellow and later in 2010 as a Senior Researcher in the department of Molecular Medicine. During this period, he led a team within the Laboratory of Cell Biology and Regenerative Medicine and focused his research on regenerating tissues and organs using stem cells for investigative and therapeutic purposes. In addition, he received training in several high-profile institutes focusing on kidney development, stem cells, and their participation in regenerative processes. In 2016, Christodoulos was honoured with the distinction of Knight of the Order of the Star of Italy for his contribution to the progress of scientific research in Italy and to Italian-Cypriot cooperation. Since 2017, he is the head of the Laboratory of Organ Regeneration and his group employ developmental biology and tissue engineering methods to produce implantable kidney tissues *in vitro*, and to regenerate damaged organs by modulating intrinsic developmental pathways.

Dr Reiner Class

Associate Director and In vitro Toxicology Lead at UCB

Reiner studied biology at the Saarland University in Germany and received a PhD in biochemistry in 1991. During his post-doc time at the Wistar Institute in Philadelphia, USA he worked on immunological questions related to HIV/AIDS as well as on developing monoclonal antibodies for cancer therapy. In 1992, he joined the faculty of Drexel University School of Medicine in the department of Radiation Oncology & Nuclear Medicine in Philadelphia where he headed the Brady Cancer Research Institute focusing on radioimmunotherapy of glioma patients. In 2002, Reiner started the biotech company SymbioTec in Germany where he headed the pre-clinical development and clinical phase I/II trials of recombinant human histone H1.3 for the treatment of leukemia. In 2006, Reiner joined the pre-clinical GLP-certified CRO Pharmacelsus GmbH in Germany to build up and head the *in vitro* toxicology department. In 2008, Reiner founded C-Square Consulting providing strategic and organisational support for pharmaceutical companies and start-ups. Since 2016, Reiner is Associate Director and *In vitro* Toxicology Lead within Development Sciences at UCB Biopharma supporting the entire portfolio with various *in vitro* tools including complex *in vitro* systems. He also leads the UCB hepatotoxicity target organ strategy-working group.

Why is the integration of 3D cellular models in drug discovery so important?

Christodoulos: One of the main barriers to developing efficient therapeutics for human diseases is correlated with the inability of existing experimental tools to effectively mimic the complex structure and function of human tissues and physiology. Animal models are often limited in their ability to mimic human conditions, specifically at the molecular and cellular levels. On the other hand, the classical human cell cultures are too simplified to predict *in vivo* conditions (such as, for example, interactions between different cell types, tissues and systems etc.), and commonly undergo pheno- and/or geno-typical changes.
Because 3D cellular platforms (e.g., organoids and organs-on a chip) can mimic better some key facets of human physiology and disease, they will come to cover this methodological gap and provide an intermediate or supplementary validation step between animals and clinical trials. Clearly, this could significantly improve our ability to predict, cure and manage human diseases in multiple ways: first, it would allow scientists to study diseases and test new therapies directly on human tissue, which would significantly improve the translatability of candidate drugs (in terms of efficacy and safety). Second, human tissue

» The development of animal and human 3D cellular models provided a path for more physiological testing with a better predictivity and translatability to animals and humans «

could be used to study diseases and test therapeutic efficacy in a personalised manner. For example, developing an organ-on-a-chip that could be derived from the patient's own cells (e.g., using reprogrammed iPSCs), could help understand the patho-physiological features of the disease in a patient-specific manner, compare different pathological genotypes and formulate a patient-tailored therapeutic strategy.

Reiner: Although science continues to make significant progress in the field of *in vitro* cell-based assays, the main reason for drug attrition remains unwanted toxicity which could not always be predicted in pre-clinical testing. Three major reasons for this gap can be identified: [1] pre-clinical toxicology testing is done in animals and not human, [2] most of our *in vitro* cell-based test systems rely on tumour cell lines and not primary or stem cell-derived healthy cells, and [3] the vast majority of our cell-based tools are done in 2D which does not adequately mimic the more complex three-dimensional situation of the complex cellular network found *in vivo*. In fact, one could make the provocative statement that we are testing in the wrong cell, in the wrong species and in the wrong format. However, the development of animal and human 3D cellular models provided a path for more physiological testing with a better predictivity and translatability to animals and humans which should improve pre-clinical testing.

What is unique about 3D cellular models/different from other available models?

Reiner: Cells cultured in 3D have many more cell-to-cell interactions compared to 2D which is much closer to the *in vivo* situation. In conventional 2D models, every cell has contact to the plastic surface and only a few adjacent cells. About half of the cell surface is exposed to the media and cell morphology is more flattened. In other words, a 2D culture is very artificial and not representative of the natural environment of a cell in the tissue. Moreover, the flexibility of mixing different cell types to create a more interactive cellular network is greater in 3D compared to 2D simply because the third dimension is added. It has been shown e.g. for

3D hepatocyte liver microtissues that gene expression and expression of CYP enzymes and transporters is much more stable and closer to the *in vivo* situation. Also, primary hepatocytes in 3D maintain viability over several weeks while 2D cultures start to lose their phenotype soon after plating. This capability of long-term culture also opens opportunities to dose cells repeatedly over several days or even weeks which better mimics a chronic dosing regime in patients.

» The flexibility of mixing different cell types to create a more interactive cellular network is greater in 3D compared to 2D. «

Christodoulos: 3D cellular systems have several unique features compared with animal models and traditional 2D cultures. First, compared with animal models human 3D cellular models enable more opportunities for experimental manipulations because they are isolated multicellular systems, are amenable to real-time imaging techniques, and, most importantly, enable the study of human developmental processes and pathogenic pathways that are not easily accessible or inadequately replicated in animal models.

Compared with monolayer traditional cultures, 3D cellular models (like organoids and organs-on-a-chip) contain more than one cell type. This enables more physiological modelling, as they can replicate various aspects of the disease, especially when the pathogenesis involves interactions between different cell types – as commonly occurs in kidney diseases. It is also very important to stress that cells in 3D culture systems maintain genome stability and phenotype better compared with primary cultures, which makes them suitable for biobanking and high-throughput screens. For example, primary cultures of tubular cells exhibit limited proliferation capacity, while the expression of drug metabolism genes decreases quickly during culture, limiting their application in drug screening.

What are your expectations/key aspects for 3D cellular models used in drug discovery?

Christodoulos: Currently, 3D cellular systems can be used to model only certain designated properties of human organs and physiology.
In the near future we may be able to partially integrate other key players of human physiology and pathobiology – such as the immune system or neural cells – in the existing microphysiological systems. I know that both academia and the industry, as well as public funders, have made huge efforts in this direction and there are already very promising results.

Nevertheless, replicating complex organ architectures and multifaceted interactions between tissues (such as those found *in vivo*), will take a longer time to achieve. To do this, we should first significantly improve our capacity to meaningfully replicate human organ development *in vitro* by providing the necessary biochemical, mechanical and physical cues. The closer we get to replicating normal organogenesis, the more realistic the culture systems we produce in the lab will be.

To this end, next-generation engineering methods will be crucial for years to come to provide better spatial and temporal control of differentiation and patterning signals, and they will gradually enable the engineering of more realistic and, hopefully, more reproducible tissue architecture and composition.

Reiner: A large number if not most testing systems for *in vitro* potency, efficacy and toxicity are based on 2D cell cultures using tumour cell lines. While there are clear advantages of those models such as reproducibility, robustness, low complexity, and low cost, the translatability gap remains a challenge. 3D cellular models used in drug discovery are expected to fill this gap by providing data

with more *in vivo* relevance. My expectation is that 3D models using non-tumour cells (e.g. primary cells or iPSC-derived cells) will gradually replace traditional 2D models and that those models become more accessible and cheaper to use. The challenge remains for 3D models that do not self-assemble like liver microtissues. For those, I expect more investment into research and development of *in vitro* strategies to better replicate the tissue development occurring *in vivo*. Once developed, said systems need to be qualified, automated and offered as fee-for-service to drug discovery functions within the pharmaceutical industry. I also expect that 3D models will be increasingly used for submissions to regulatory authorities and that, ultimately, they will be accepted as a suitable replacement for animal studies.

Where do you see Evotec's key contribution?

Reiner: Companies like Evotec play a very critical role in drug development since the business model of most pharmaceutical companies is based on outsourcing all routine assays to Contract Research Organisations (CROs). I see four major contributions of a CRO like Evotec: [1] provide services using complex 3D *in vitro* models that are high quality, robust, reproducible, low cost with quick turnaround times, and [2] work with pharmaceutical companies to identify needs, [3] develop novel tools to address the needs, and [4] work with other companies and regulatory authorities to generate rules for acceptance and quality guidelines. This requires heavy investments in several areas such as automation and qualification and needs a closer interaction between stakeholders. A CRO like Evotec needs to transform from a service provider to an extended work bench of the customer. A very close interaction between customer and CRO will be key to success. In my opinion, quality and cost are not the driving force behind selection of a CRO since most provide high quality work at a reasonable cost. What discriminates CROs is their ability to interact with the customer and work as a partner rather than a service provider. Finally, I expect a good CRO to drive development, publish scientific findings in peer-reviewed journals, actively participate at conferences and support pharma companies in addressing bespoke issues.

Christodoulos: Normal renal function depends not only on cellular homeostasis, but also critically on the architecture of both the individual cells and the organ. Nephrons (the filtering units of the kidney) work through a complex multi-step process that involves a specialised microvascular bed (containing fenestrated endothelial cells,

» 3D cellular models used in drug discovery are expected to fill the translatability gap by providing data with more *in vivo* relevance. «

highly specialised podocytes, and mesangial cells) that produces the primary filtrate, and an epithelial tubule that returns needed substances to the blood and pulls out additional waste. Unfortunately, classic monolayered 2D cultures cannot efficiently model these complex structures and processes, while animal models lack some key molecular and cellular features of the human nephron. Evotec may have been one of the first industrial players who decided to do something about this problem and made the strategic decision to face it. They built up a group of experts (which I had the fortune of participating in) and eventually developed an in-house podocyte-on-a-chip platform using iPSC-podocytes and microfluidic chips. Along with this platform, they also established a range of functional assays to quantify filtration barrier integrity, predict drug toxicity and perform mechanistic studies in the context of chronic kidney disease.

Apart from the hands-on impact of this advance – which will facilitate Evotec's efforts to discover new medicines for chronic kidney disease – these studies also provide proof-of-concept of how 3D microphysiological systems can be used in routine biomedical research.

When do you think patients will ultimately benefit from advanced 3D cellular models?

Christodoulos: Being able to grow human tissues in a dish, from the early developmental stages to having a mature tissue or organ, will revolutionise modern biology and biomedical research. 3D models (like organoids and organs-on-a-chip) can be used to model normal development and diseases, and for personalised drug testing and discovery studies. Although, a lot of work remains to be done in order to fulfil all these expectations, some of the possible applications are already being used in the arena of drug testing (e.g., tumour metastasis and vascularisation models). For example, we have conducted drug testing in engineered tissues from patients with polycystic kidney disease, and colleagues around the world are using 3D microphysiological systems for investigational purposes and initial pre-clinical screening (of course this in parallel with and supplementary to animal studies). From this perspective, patients are already benefitting indirectly. However, I believe that in the future we may also be able to use 3D systems in clinical practice, for instance as tools to decide which drug would be the most efficient as in the case of expensive antibody-based therapies or which chemotherapies should be used in a specific patient.

» All pharmaceutical companies I'm aware of already use complex 3D models in their routine to guide decisions, understand Structure-Activity-Relationship (SAR) and design better compounds. «

While working in this direction, one of the most important challenges for researchers in the field is how to develop robust, reproducible, and replicable technologies that can easily be transferred to different laboratories and reliably used on a large scale.

Reiner: I believe that patients already benefit from advanced 3D models. All pharmaceutical companies I'm aware of already use complex 3D models in their routine to guide decisions, understand Structure-Activity-Relationship (SAR) and design better compounds. The ultimate benefit for the patient will be if we are able to use novel platforms (e.g. organs-on-chip that connect multiple organs / tissues with a flow) and cells directly derived from the patient. We have seen an increased use of iPSC-derived patient cells for target discovery and drug development across the industry. However, standardising methods, finding and agreeing on quality criteria, automating the process, and regulatory acceptance remain an unsolved challenge. Finding new medicines for our patients is getting increasingly complicated, costly, and time consuming but for most drug developers, time will probably become the most precious asset. Getting to the market earlier will allow for earlier access of patients to therapeutics, longer use of patents, and subsequently increased revenues.

CHAPTER 04

5 QUESTIONS

DR MAGALI FERRO & DR STEPHANIE RYDER

SHORT SUMMARY OF SCIENTIFIC CAREER

Magali received her PhD in bioengineering from Ecole Nationale des Mines de Saint-Etienne, France, designing and developing microengineered tissue-barrier models that incorporate impedance measurements in real time to assess barrier integrity in longitudinal toxicity studies. During this period, Magali was awarded a visiting Scholar Fellowship from Stanford University to further develop a 3D model of the human blood brain barrier to predict drug toxicity. Her proven track record in the field of 3D cellular models of human tissues includes research papers in Advanced Biosystems, Lab on a Chip and Science Advances. In 2018, Magali joined the department of Engineering at Cambridge University, UK, as a postdoc working collaboratively with Evotec to build a Kidney-on-a-chip model and help unravel the impact of the cellular microenvironment on the establishment of a glomerular filtration barrier. Magali joined Evotec in 2020 as a research scientist where she is responsible for developing, qualifying and using organ-on-a-chip systems, for example to model kidney diseases and discover new therapeutics.

SHORT SUMMARY OF SCIENTIFIC CAREER

Stephanie studied Biochemistry at the University of Liverpool, UK, later receiving her PhD in toxicology from the University of Liverpool. During her PhD Stephanie worked with AstraZeneca developing 3D cardiac microtissue models to study the role of cardiac endothelial and fibroblast cells in drug-induced cardiovascular toxicity for which she received their Innovation & Achievement Recognition award. In 2014, following her PhD, Stephanie joined Cyprotex, an Evotec company, as a Senior Scientist within the toxicology team. Here her focus was to expand her expertise in 3D microtissues into the development of cardiac, liver and brain microtissues for the application of high-throughput high-content imaging assays. Cyprotex now have market leading expertise and offerings in the field of 3D high-content imaging. In 2016, Stephanie was shortlisted for the BioNow Promising Biotechnologist of the year award. Stephanie was appointed as Toxicology Team Leader at Cyprotex in 2021 taking on responsibility for the operational delivery of client work as well as driving future scientific developments.

1 The use of complex cellular models of human organs in drug discovery holds great promise. Could you summarise where you see the greatest potential but also the greatest challenges?

Magali: Over the last decade, tremendous progress has been made in tissue engineering and the development of complex 3D cellular models for drug discovery applications. Academic and industrial institutions continuously generate proof-of-concept data providing evidence that for some studies, 3D models have the potential to mimic critical biological functions and better predict the human response to new drug candidates or advanced therapies than existing validated models. With the emergence of novel therapeutic modalities, the use of more physiologically relevant systems could significantly support the selection of clinical candidates at advanced drug development stages by predicting potential human-relevant organ toxicity, drug availability and drug efficacy that are difficult to mimic in simple cell-based screens and for which animal models are not always relevant.

However, considering the high rate of complex 3D cell model developments, covering a wide range of human tissues and organs, their adaptation in the pharmaceutical industry is relatively slow. Before being used in drug discovery programs, any newly developed model needs to be thoroughly characterised and qualified, in the specific context of use, to avoid the risk of generating misleading data that might compromise the progress of promising compounds into the clinic. This is one of the key challenges, as drug discovery companies must invest time and money to move from established routine trials to new platforms and trust that these complex 3D models will yield relevant and informative data. The integration of complex 3D cellular models in drug discovery programs relies on matching the biology of the model with a specific purpose, ensuring reproducibility of the results and, if possible, demonstrating model predictability using well-characterised tool compounds.

Stephanie: As Magali discusses, huge progress has been achieved in the development and characterisation of more complex 3D models suitable for pre-clinical drug development. However, one understated challenge of 3D models is the development of methods capable of analysing these models and coping with their 3D nature. Traditionally, *in vitro* toxicology assays are set up for 2D monolayer models with a single plane. For example, with the advent of 3D microtissue models this has meant our techniques for high-content imaging had to drastically adapt; light exposure, microtissue locating, imaging slicing, maximum projections are now all integral parts of our 3D high-content imaging platforms – a complexity not required with monolayer models. In addition to this, many biochemical assays and transcriptomic techniques have had to adapt and increase in strength and/or sensitivity in order to cope with additional lysis requirements of 3D models. While automated liquid handling robots now have the added challenge of handling non-adherent cells when aspirating and dispensing. Without advancements in these analysis and handling techniques alongside the development of more *in vivo* relevant 3D models, we will ultimately limit their capability and capacity to predict compound effects accurately *in vitro*.

2 How can Evotec's 3D cellular models contribute to better predict the safety profile of new drug candidates?

Stephanie: Traditionally *in vitro* safety assessments have relied heavily on immortalised cell lines in a restrictive 2D organisation that poorly recapitulate the *in vivo* cellular physiology; resulting in poor prediction of later *in vivo* toxic events, poor drug attrition early in the development pipeline and ultimately wasted cost and time. Evotec/Cyprotex have developed our in-house 3D models with the overall aim of bridging this gap between *in vitro* and *in vivo* findings. We appreciate the multi-cellular complexity essential to the normal functioning of organ tissue and the requirement to have systems with more points for disruption but also capability for physiological response/adaptation. Hence our 3D models are multicellular and formed using round bottom low adhesion plates which promote the free migration of cells into spheroidal tissue structures. One major benefit of this approach is that it allows the high-throughput

generation and analysis of these microtissues so that we can offer advanced *in vitro* screening at the high-throughput level required early in the screening phase of drug development programs. Our models themselves combined with our approach utilising multi-parametric high-content analysis allows mechanisms of toxicity to be detected prior to gross cell death. Furthermore, recent advances in the field of high-throughput transcriptomics have allowed the advent of Evotec's PanOmic's safety initiative to develop a transcriptome safety database. This utilises our HT-transcriptomics system Screenseq™, with robust automated cost-efficient technology able to detect up to a million mRNA molecules per well, optimised for a diverse set of cell systems, including more than 25 cell models and 3D microtissues. This technology used in combination with our sophisticated EVOpanHunter (AI/ML) analysis platform will further advance our prediction of potential *in vivo* safety liabilities meaning that well-informed decisions and risk assessment can be made early in pre-clinical drug development; saving time, effort and cost (you can find more information about it in DDup 10).

3 How can Evotec's 3D cellular models contribute to disease understanding and aid the discovery of new compounds?

Magali: About 60% of drug attrition is due to a lack of drug efficacy. This observation underlines the poor relevance of many disease models and makes the establishment of human-relevant disease models an urgent need. To respond to this need, in particular in the field of kidney diseases, Evotec is developing multiple complex 3D cellular models of the human kidney, including organoids and organ-on-a-chip systems. These models cover multiple contexts of use, such as nephrotic syndrome with acute podocyte injury and autosomal dominant polycystic kidney disease. The design and qualification of targeted assays, with endpoints in reference to the conventional human endpoint, allows us to use these models in early drug discovery projects to explore new targets and treatment paradigms not covered by existing, validated models. For example, in this DDup we report a glomerulus-on-a-chip designed to mimic the glomerular filtration barrier that can be disrupted in a diabetic nephropathy context. The model reflects relevant disease states by capturing elements of the podocytopathy cascade, including cytoskeleton rearrangement, podocyte detachment from the GBM, podocyte hypertrophy, and increase in albumin level in the primary filtrate. We feed our 3D kidney models with highly characterised iPSC-derived cells to [1] ensure a constant source of good-quality cells, [2] open the possibility to use patient-derived cells for precision medicine and [3] embrace the inter-individual differences in response to pharmacological treatment, often observed in clinical stages. Lastly, our models are currently being qualified to discover translational biomarkers that could potentially inform us on target engagement and disease modification early on.

4 What is Evotec's approach to integrate complex 3D cellular models in the drug discovery pipeline?

Stephanie: At Cyprotex, we believe our 3D microtissue models have the ability to widen the pre-clinical scope traditionally covered by more complex 3D models. In the past, initial chemical screening phases of drug development have been performed utilising basic cell line monolayer models in simple single assay type analysis. We have developed our microtissues with various key qualifications in mind: (a) improved *in vivo* relevance, (b) better longevity, (c) lower cell number requirements, (d) high-throughput plate formats i.e. 96 and 384 well, (e) applicable to multi-parametric analysis techniques and finally (f) compatible with automated liquid handling. By fulfilling each of these criteria, we now have multiple organ-type microtissues available that not only show better recapitulation of *in vivo* tissues and therefore improved toxicology predictions; but also allow reduced cell requirements, chronic dose settings, high-throughput handling and multi-parametric data acquisition. Thus reducing assay costs and widening the scope of these models into earlier phases within the drug development pipeline.

Magali: Drug discovery is a stepwise process in which human-relevant models are required at all phases to ensure the selection of a safe and efficient drug candidate to advance in the clinic. Each step has specific needs in terms of throughput capacity and complexity of the model, and therefore multiple models should be used to provide the required data. In the screening phase, the choice of model is mainly driven by its high-throughput (HT) capacity. However, we prioritise complex 3D cellular disease models at the target identification, target validation and candidate selection phases, while the throughput of the model is secondary. Evotec developed multiple human-relevant 3D kidney models covering the different levels of complexity and throughput capacity while ensuring model-to-model translatability across the drug discovery stages. For example, to support drug discovery studies focusing on podocyte-associated diseases, we provide three models with increasing levels of complexity: podocyte monolayer, 3D podocyte microtissues and podocyte-on-a-chip system. Highly characterised human iPSC-derived podocytes feed all models to facilitate the model-to-model translatability and to better predict the clinical outcome. The podocyte monolayer and microtissues are compatible with HT technologies and used to predict cell structure, and transcriptomic profile changes. The podocyte-on-a-chip system is more complex and used at a lower throughput to gain a mechanistic understanding of the target mode of action. To ensure the delivery of high-quality data, before using any of our models for drug discovery studies, we qualify them in response to a specific purpose by evaluating their suitability to mimic healthy and disease phenotypes and functions. In addition to clarifying the context-of-use of the model, we establish the utility and domain of the assays we develop by clearly defining how to interpret positive and negative responses. By following this framework, as suggested by pharmaceutical industries and regulatory agencies, we believe that we will facilitate the integration of complex 3D *in vitro* models into the drug discovery pipeline.

5 What other models are on the horizon?

Magali: In response to the high drug attrition rate and in alignment with the 3Rs (replace/reduce/refine) rules for animal testing, Evotec is investing in emerging technologies, such as engineered 3D *in vitro* models, for example microtissues, organoids and microphysiological systems and incorporates them into our state-of-the-art drug discovery pipelines. Here, these novel 3D technologies are developed, qualified, and used to model human diseases, discover new therapeutic pathways, and predict patient drug efficacy and safety. In addition to model kidney diseases, Evotec is interested in developing complex 3D *in vitro* models of the retina and the liver.

Stephanie: One major aspect that is currently still missing on a high-throughput assay amenable format for early drug screening is the use of (a) organ-on-a-chip type systems whereby different tissue types within an organ can interact and communicate but also (b) human-on-a-chip type systems which allow different organ types to interact. These systems would further bridge the *in vitro* to *in vivo* gap by allowing organ tissue interactions and communications which will ultimately be key to the refinement of our *in vitro* toxicology predictions.

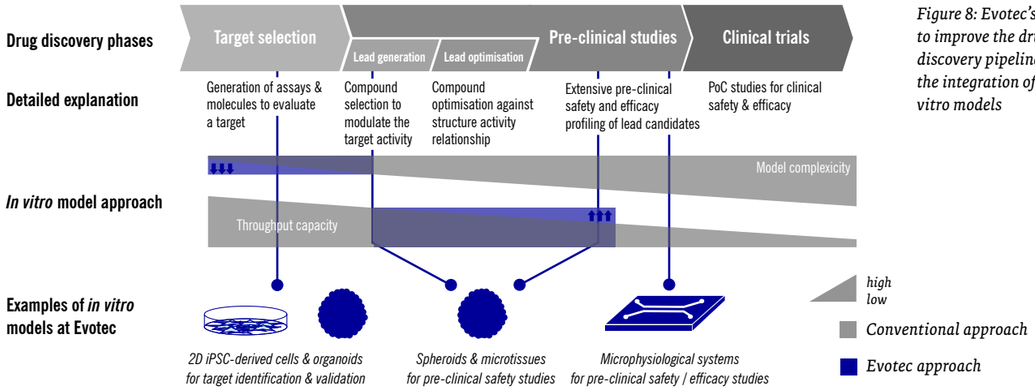

Figure 8: Evotec's approach to improve the drug discovery pipeline through the integration of 3D in vitro models

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