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Phenotypic screening with 3D cell-based assays

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chapter 7

General discussion and future perspectives

Tijmen H. Booij



Three-dimensional cell culture assays

In order to overcome biological limitations of traditional *in vitro* disease models as discussed in **chapter 1**, three-dimensional (3D) cell culture techniques were developed to bridge the gap between these *in vitro* models and the *in vivo* situation.^{1,2} However, while 3D cell culture techniques provide biological advantages over 2D cell culture models, these are often not exploited to their full potential (**chapter 2**). For example, while more biologically relevant, these *in vitro* models have often not been adapted for routine compound screening due to problems associated with scalability and increased cost of tissue culture reagents.³ As a result, 3D cultures are generally employed to validate findings that were originally observed in two-dimensional (2D) cell culture context, prior to *in vivo* evaluation. While the 3D cell culture models can in this context eliminate false positives prior to animal experiments, it is likely that the lack of relevant biological features of 2D cell cultures causes an increased false-negative rate in the initial observations in 2D cell cultures (**chapter 1**). Advances in laboratory automation have enabled large-scale preparation of 3D cultures for high-throughput screens (HTS), and throughout this thesis I applied this technology, coupled with high-content analysis, to perform screens using 3D cultured micro-tissues.

High-Content Screening (HCS)

Simple biochemical or colorimetric measurements of compound efficacy on cell cultures (e.g. ATPlite or MTT assays) often only provide limited information on the biological context.⁴ While an ATPlite measurement can provide very relevant measurements of ATP content and related general cell health status,^{5,6} a molecule that kills cells is not necessarily a good drug. For example, in **chapter 6**, I described the potent efficacy of antineoplastic drugs at inhibiting 3D cyst growth in the context of polycystic kidney disease (PKD). In the context of cancer, a DNA-intercalating- or otherwise growth-inhibitory drug may be effective at treating or delaying the disease. In the context of PKD, a molecule that inhibits cell growth is also likely to arrest cystogenesis, because increased cell proliferation is an important driver of cyst growth. Importantly, because PKD is a disease with a slow progression, such a therapy is likely to come with severe side effects that limit its use as a drug for PKD patients.

An alternative to using traditional biochemical efficacy measurements is using phenotypic information, often termed high-content analysis (HCA) or HCS in the context of screening⁷⁻⁹ (**chapter 2**). Although HCA currently is more commonly used together with 2D cell culture assays, phenotypic information can also be derived from the individual 3D micro-tissues,¹⁰ where tissues are individually imaged and quantified against a pre-defined set of phenotypic descriptors for various possible characteristics (branching morphology, roundness, size, nuclear elongation, etc.).¹¹⁻¹³ An advantage offered by HCA is that it is possible to conduct drug discovery at a target-agnostic level,⁷ thus opposing target-based drug discovery. Additionally, information on cellular phenotypes can be used to identify potential off-target effects of molecules, or even cytotoxic effects. For example, in **chapter 6**, phenotypic information was used to exclude potentially toxic molecules, and this indeed limited the selection of known antineoplastic

compounds. Throughout this thesis, I applied HCS on different cellular assays, to discriminate selective inhibitors from non-selective inhibitors of receptor tyrosine kinases, to identify modulators of cystogenesis and to find drug candidates that can effectively slow down the growth of renal cysts.

Discriminating small molecule inhibitors by target

In **chapter 3**, I used this high-content analysis, also termed phenotypic profiling, to discriminate epidermal growth factor receptor (EGFR) and c-Met receptor tyrosine kinase inhibitors in the context of tumour cell invasion. Using phenotypic profiling, I could selectively classify molecules that inhibited EGFR or c-Met, and also identified dual inhibitors for these receptors. Interestingly, off-target effects by molecules that inhibited tumour cell invasion through different mechanisms were also observed. Therefore, using phenotypic profiling it is possible to screen for the inhibition of disease phenotypes, while target-based screening can also be performed. One limitation to this approach is that target discrimination relies on the phenotypic manifestation of target inhibition, or activation. However, this phenotypic target-based approach can be of particular interest for the identification of off-target effects,⁷ as these are not commonly captured using traditional biochemical approaches.

Identification of druggable targets for polycystic kidney disease

In **chapters 4 and 5**, a 3D cyst screening platform was used to screen molecule libraries containing kinase inhibitors with pre-determined molecular targets. In these chapters, I related the effects of these molecules to their known molecular targets to identify pathways relevant in cyst growth. This approach led to the selection of targets such as mammalian target of rapamycin (mTOR), which has been extensively described for PKD¹⁴⁻²⁰ and can therefore confirm the relevance of this approach. Additionally, we discovered that inhibitors of cyclin-dependent kinases (CDKs) potentially inhibited cyst growth, which is also in line with earlier observations for roscovitine.²¹ Although very potent, PCA analysis could discriminate roscovitine from unstimulated controls in **chapter 4**, indicating that the phenotype of the cysts is altered compared to unstimulated cysts. While the activity of roscovitine in other PKD models has been confirmed, roscovitine has not yet entered clinical trials, possibly related to safety concerns for the use of this type of drugs for PKD.²²⁻²³ This potential for causing side effects is also illustrated by the results I presented in **chapter 5**, where many CDK inhibitors that inhibited cyst growth also affected morphology of 4T1 tumouroids, indicating that these inhibitors do not exclusively target cystogenesis, but may have a broader activity spectrum.

Interestingly, the results presented in **chapter 4 and 5**, and also in **chapter 6** (supplemental figures 2 and 3), have shown that several insulin-like growth factor receptor-1 (IGF-1R) inhibitors delayed cyst growth in our assay. The IGF-1R is a receptor for insulin-like growth factor (IGF) and its signalling is known to contribute to cell proliferation and survival through PI3K, Akt, mTOR and P70S6K.²⁴⁻²⁹ The involvement of IGF-1 in PKD progression has been described previously by other groups,³⁰⁻³² and IGF-1R inhibitors may present an interesting therapeutic option of PKD due to the increase in

IGF-1 expression observed with progression of cystic lesions.^{27, 31} Of note, the IGF-1R inhibitors tested here may also have affinity towards other receptors such as the insulin receptor, which can also bind ligand IGF,^{27, 33} and it is therefore necessary to confirm the involvement of the IGF-1R, potentially using function-blocking antibodies raised against the receptor or by downregulation of receptor expression. The low severity of the side effects (e.g. hyperglycaemia, fatigue, nausea) observed of IGF-1R inhibitors in clinical trials to treat cancer,³⁴ and the absence of dose-limiting toxicity,³⁵ further supports the potential of IGF-1R inhibitors for the treatment of PKD.

Similarly, in **chapter 4 and 5**, I described the efficacy of inhibitors for HER2, an important molecular target in aggressive breast cancer,³⁶ at inhibiting cyst growth in our 3D cyst assay. HER2 was identified as a potential target for PKD more than a decade ago, but was not followed up since.³⁷ Whether HER2-targeted therapy would also be effective in PKD patients would therefore be unclear. HER-2 targeted therapy (generally monoclonal antibodies against HER2 rather than small molecule inhibitors) has been tested in the clinic in the context of different types of cancer, most commonly breast cancer, and is generally combined with chemotherapeutic drugs,³⁸ making it difficult to judge the safety profile, but the most common reported side effects of pertuzumab (a humanized monoclonal antibody against HER2), include diarrhoea, weakness, nausea and rash.³⁹ Although trastuzumab, a monoclonal antibody against HER2 was associated with cardiotoxicity, this was not observed for novel HER2-targeted therapies.⁴⁰ Neratinib, an oral small molecule HER2 inhibitor is also reported to cause diarrhoea,⁴¹ and this may be the result of the presence of ErbB-class receptors on intestinal epithelial cells.⁴¹⁻⁴²

Finally, the results presented in **chapter 4** suggest the importance of spleen tyrosine kinase (Syk) in cyst growth, which has not been previously reported. Syk plays an important role in the immune system⁴³ and has mostly been implied as a target for immune disorders such as asthma⁴⁴ and rheumatoid arthritis,⁴⁵ but has also been proposed as a target in cancer.⁴⁶ However, because of its role in signalling in B-cells, mast cells, macrophages, neutrophils, platelets and erythrocytes,⁴³ targeting Syk for the treatment of PKD may potentially cause side effects that outweigh its use for treating PKD.

While the drug targets described in this section are known to be inhibited by the molecules in the tested compound libraries, it is likely that these molecules also have other molecular targets at the concentrations tested, and it will therefore be important to measure protein phosphorylation levels after compound treatment to ensure that the targets are affected. Additionally, for several receptor tyrosine kinases, such as HER2, function-blocking antibodies were designed that may be more specific to the target. These antibodies may be used to confirm the efficacy of target inhibition on cyst growth.

Finding drug candidates to treat polycystic kidney disease

Currently the only available drug for the treatment of PKD is tolvaptan. While this compound can slow down the growth of renal cysts, its use may be limited because of side effects associated with diuresis. This illustrates that novel therapies are still required.

Chapter 6 described the identification of pyrvinium pamoate and celastrol as inhibitors of cystogenesis *in vitro*. Both compounds were initially selected after a phenotypic screen with 2320 compounds on 3D-cultured mouse inner medullary collecting duct cells with reduced levels of *Pkd1* expression. While this screening setup was highly convenient to screen large compound libraries due to fast cystogenesis and swelling, the choice of a murine collecting duct cell line and also the rapid cystogenesis can potentially limit the relevance of the *in vitro* findings to human disease. Firstly, the cysts in human polycystic kidney disease develop slowly over time, and not in a few days. It is therefore a possibility that proliferation-driven processes are more important in this 3D cyst culture model, compared to human PKD, which could also explain the identification of many antineoplastic molecules as cyst growth inhibitors. Secondly, the cysts developing in PKD do not solely originate from the collecting ducts, but from all segments of the nephron.⁴⁷ It is therefore possible that the effects observed from pyrvinium pamoate and celastrol are nephron-segment specific, potentially limiting therapeutic potential. Finally, the choice of using murine cells may complicate translation of our findings to PKD patients.

Since we were aware of the shortcomings of using a rapid murine *in vitro* model for PKD, the effects of pyrvinium pamoate and celastrol were evaluated in a mouse *in vivo* model for PKD. We found that pyrvinium pamoate displayed a lack of activity in this model and we therefore did not investigate this compound further. In contrast, the *in vivo* activity of celastrol confirmed the *in vitro* discoveries and celastrol could therefore present an interesting therapeutic opportunity. Further supporting the use of celastrol for the treatment of PKD is the finding that two close analogues of celastrol, dihydrocelastrol and dihydrocelastryl diacetate, also induced similar cyst growth inhibition profiles *in vitro*, although the effects of these compounds have not been evaluated *in vivo* due to lack of commercial availability and the low number of scientific publications covering their effects.

Celastrol is a molecule isolated from the thunder god vine, also *Tripterygium Wilfordii*. This vine is promoted in traditional Chinese medicine for use in rheumatoid arthritis,⁴⁸ but its use is not recommended due to safety concerns.⁴⁹ It is important to mention in that context that *Tripterygium Wilfordii* contains many bioactive molecules (~100), potentially explaining the side effects associated with its use.⁵⁰⁻⁵¹ Celastrol has not been previously linked to PKD, but has been implied for several other indications.⁵¹⁻⁵⁶ In **chapter 6**, we evaluated the effects of celastrol on several PKD-associated signalling pathways (pCREB, pS6 and pSTAT3), but observed no effect on these targets. As several publications have previously suggested, celastrol may have many molecular targets such as HSP90/Cdc37,⁵⁷⁻⁵⁸ NFκB⁵⁹ and Nrf2.⁶⁰ HSP90 inhibition has

previously been shown to be an effective strategy to reduce cyst growth *in vivo*, probably because many pathways activated by *PKD1* or *PKD2* inactivation are regulated by HSP90.⁶¹ Additionally, oxidative stress is observed in all stages of ADPKD⁶² and may be a general mechanism in the pathogenesis of PKD.⁶³ Inhibition of NFκB and activation of the Nrf2 by celastrol and the associated antioxidant response could therefore be an important mechanism by which celastrol can inhibit the growth of cysts, although whether this is also the case in our *in vitro* and *in vivo* model remains to be determined. In addition to these targets, celastrol is also known to be able to interact with thiol groups in many proteins^{58, 64} as is the case for many plant extracts (e.g. curcumin⁶⁵), probably giving celastrol a very broad target space that has not yet been completely unravelled. Interestingly, such a broad target specificity also makes it unlikely to identify similar molecules in traditional target-based drug discovery approaches – thereby favouring phenotypic screening techniques. As currently the mechanism by which celastrol inhibits cystogenesis in our model is unclear, an analysis of the transcriptome of 3D-cultured cysts treated with celastrol could shed light on the pathways that are affected.

Very importantly however: a broad target specificity can be beneficial in diseases where many signalling cascades are effected (such as PKD), but it is also a concern for safety, as it is more likely that other organs and physiological processes are affected. Increased insight in the pathways that are affected by celastrol by transcriptomics, as well as the use of fluorescent reporters for cellular stress pathways could be used to predict potential toxicity.⁶⁶⁻⁶⁷ Fortunately, in our *in vivo* experiments, celastrol did not appear to cause many side effects, except for a reduction in body weight between P13 and P18, as was also reported by others for 1mg/kg/day.⁵² While such a reduction in body weight can be related to dose-limiting toxicity or effects on other organs than the kidney, the reduced bodyweight after celastrol treatment are more likely related to its known effects as a leptin sensitizer and its potential use in the treatment of obesity.⁶⁸⁻⁶⁹ Reduced food intake as a result of this effect of celastrol may have biased our findings as food restriction is also known to ameliorate PKD progression.⁷⁰ However, it is unlikely that the effects observed on *in vitro* cultured cysts are the result of this mechanism. One limitation of the *in vivo* PKD model used in **chapter 6** is the rapid disease progression. Because these mice develop renal failure within a few weeks, which does not correspond with the slow disease progression in humans, the efficacy of celastrol in a chronic disease *in vivo* model remains to be determined and will also be critical to detect potential safety concerns associated with long-term administration. Whether celastrol is likely to become a drug for PKD, while maintaining a desirable efficacy profile is therefore, despite the promising results presented in this thesis, still uncertain.

As a result of the many signalling pathways that are deregulated in PKD as described in **chapter 1**, one could argue that a single drug cannot be sufficient to prevent cystogenesis. An interesting approach could therefore be to test drug combinations, for example from the other identified active compounds in **chapter 6**, on 3D cell culture models of cystogenesis. This may potentially identify synergistic effects, allowing for dose reductions of the individual compounds.

The current availability of 3D cell culture matrices and the ability to prepare 3D cultured cells in multi-well plates using automated liquid handlers, has improved the quality of *in vitro* PKD models over the past years, which is now likely to aid in the selection of more effective, and safer drugs. In addition, the *in vitro* selection of better drugs could eventually contribute to a reduction in the required animal experiments, although some hurdles need to be overcome to further improve these models, as discussed below.

Current limitations of 3D HCS technology and future perspectives

In **chapter 2** I discussed limitations of 3D cell culture techniques that have hampered their implementation in routine drug discovery. These difficulties include the use of natural ECM protein matrices for 3D cultures, such as collagen and Matrigel, because these suffer from batch-to-batch variability and also complicate liquid handling because the reagents solidify at room temperature. Currently, synthetic hydrogels generally have limited biocompatibility compared to these natural hydrogels.⁷¹ However, several examples now exist where this type of gel can provide improved biological context due to incorporation of functional groups,⁷²⁻⁷³ and it is possible that such techniques can be incorporated in high-throughput screens and will be more accessible in the future.

Sample preparation from 3D cell cultures, such as the extraction of protein for Western blot or RNA extractions to measure gene expression levels, is also more challenging, and requires additional purification steps compared to regular 2D cell cultures. This is especially important when attempting to extract cellular proteins: Matrigel or collagen-based gels contain high concentrations of proteins that can far exceed the content of cellular proteins, thus requiring removal of proteins from the gel prior to cell lysis. Another challenge lies in performing immunostainings, as antibodies take longer to diffuse through the ECM, requiring longer incubation times and more extensive washing steps. Nevertheless, these problems can probably largely be overcome by setting up standard procedures for these techniques optimized for 3D cell cultures, although whether this is successful will also depend on the type of culture matrix.

Currently, the popularity of immortalized cell lines for high-throughput screens, such as the PC-3, mIMCD3 and 4T1 cell lines used throughout this thesis, can pose a barrier to the physiological relevance of cell culture assays, because organs are comprised of many different cell types that cannot be represented completely by a single cell line.⁷⁴⁻⁷⁵ This means more extensive validations are required to be able to translate findings on immortalized cell lines to the human body. While immortalized cell lines are generally convenient in high-throughput screens due to their ease of culturing and also because of their relative lack of heterogeneity, depending on the research question it is likely that the introduction of multiple cell types in the form of co-cultures may allow us to improve physiological relevance. However, this may also introduce more experimental variation, making implementation in HCS more challenging.

Moving away from immortalized cell lines altogether and instead using patient-derived cells is likely to tremendously enhance physiological relevance of 3D cell-based assays. For example, in the context of PKD, patient-derived induced pluripotent stem cells (iPSCs) can be differentiated to kidney cells⁷⁶⁻⁷⁸ and iPSCs have been generated with mutations relevant to PKD.⁷⁹⁻⁸⁰ A clear advantage of such PKD patient-derived cells is the biological relevance of the mutation. While most generated immortalized cell lines harbour a deletion of an entire gene or large gene fragment, these iPSC-derived tissues retain a disease-causing mutation (although these are patient-, and possibly also species specific – meaning that it may not always be possible to validate findings in animal models⁸¹⁻⁸²). Even though iPSC-derived tissues can be used for high-throughput screening,⁸³ their extensive differentiation procedures and slow growth can pose new challenges, especially for screens of 3D cultures. Therefore, to what extent such cell types can be used in routine high-content screens as 3D culture remains to be determined, but it is likely that such cell types will be used more frequently in the future as an alternative to immortalized cell lines in screening.

It has even proven possible to generate self-organising kidney organoids from pluripotent stem cells.⁸⁴⁻⁸⁵ These kidney organoids can contain many different cell lineages and can more accurately model kidney development.⁸⁶ While these organoids display increased functionality compared to single cell types, they are not currently used in high-throughput phenotypic screens. It is possible that their composition of multiple tissue types may make phenotypic screening more challenging, as the composition of individual organoids may be too variable. The presence of multiple cell types also puts additional strain on image capturing and image analysis, because it may no longer be sufficient to capture a series of xy-images per well. Instead, such organoid cultures are likely to require 3D reconstruction at high magnification to capture sufficient detail. While such organoids may therefore eventually enable screening of molecules for organ toxicity (e.g. nephrotoxicity) or kidney disease, the technology is currently still in its infancy and it is probably going to take another 5 to 10 years before such micro-tissues can be routinely used for phenotypic screens. However, an interesting application of these self-organising kidney organoids, is the development of bioengineered kidneys to treat disease.⁸⁷

In addition to the limitations posed by the cell types used, extraction of phenotypic information from 3D cell cultures is currently limited by computational power and storage capacity. Due to the vast quantity of image data that is typically generated from multi-well plates with 3D micro-tissues (50-100GB per 384 well assay plate), dedicated storage facilities and excellent infrastructure are required. Additionally, the quality of the imager and the image analysis tools can potentially limit the quality of the readout. On the upside, judging from the progress in the last ten years in storage capacity, computational power and quality of the microscopes, this barrier to the implementation of 3D HCS may soon become a thing of the past.

Conclusions

This thesis described various strategies to employ 3D phenotypic screening in a high-throughput setting; ranging from the identification of new drugable targets to the selection of lead molecules. While 3D cell culture-based high-content screening is not yet routinely applied in drug discovery campaigns, the improved biological relevance of these assays is likely to improve translatability of results to the clinic and may drastically reduce the animal experiments that are currently required in drug research, and reduce barriers for more personalized medicine.

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