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UNLOCKING TOMORROW'S CURE FOR CANCER: *IN VITRO* MODELS FOR ONCOLOGY DRUG DEVELOPMENT

2D, 3D CELL MODELS, AND TUMOR ORGANOIDS UNDER THE MAGNIFYING GLASS

Contents

1. Executive Summary

Over the last decade, oncology drug development has been marred by the high attrition rates of new anti-cancer agents. The high failure rate is attributed to both poor translatability of preclinical models and their suboptimal use at different stages of drug development. Two-dimensional (2D) cell culture models have traditionally been used to interrogate disease mechanisms and have provided significant contributions to our current understanding of cancer biology. However, several published reports have highlighted the poor predictivity of patient response by 2D *in vitro* models.

As a result, patient-derived models including primary cells and *in vivo* patient-derived xenografts (PDX), which more faithfully recapitulate original tumor pathophysiology, have increasingly been adopted to evaluate drug response. However, these models each come with their own challenges, such as limited availability of patient tissue, time, and cost to develop.

Organoids represent a revolutionary approach to drug development because they offer *in vitro* predictivity of patient response previously only seen with *in vivo* PDX, faster developmental time compared to *in vivo* models, and enhanced robustness and reproducibility compared to standard 3D *in vitro* systems.

With the development of tumor organoids, opportunities for *in vitro* investigation of new anti-cancer agents have significantly broadened. Drug developers can now adopt a range of *in vitro* model types from 2D models to dissect signaling pathways and more advanced patient-derived 3D models to predict patient response.

Throughout drug discovery, the choice of the right preclinical *in vitro* model is critical for decision making and translatability. This report provide an overview of the optimal uses of 2D cell models, 3D cell models, and tumor organoids in cancer research. It will further discuss the different applications of patient-derived tumor organoids and how they are revolutionizing cancer drug discovery and development.

In this report, you will…

- Discover the key features of 2D and 3D cell models in the context of cancer drug development
- Learn the advantages, applications, and limitations of current cell-based models
- Study the benefits of tumor organoids in translating preclinical results into humans
- Explore the different applications of organoids in cancer drug development
- Find out how the use of organoids and 2D and 3D cell models will develop in the future

2. Challenges in *In Vitro* Cancer Drug Development

Approximately 60% of novel anti-cancer drugs fail in clinical trials and only about 5% of anti-cancer agents succeed to get from first-in-human studies to marketing authorization, which is a lot lower than for other disease areas[1,2].

As a result, there is a pressing need for adopting more translatable, patient-relevant models at earlier stages in oncology drug discovery and development[2]. One step towards greater translatability in preclinical cancer research is the development of more advanced *in vitro* models, such as those directly derived from patient tissue (primary cells), which can be grown in 2D or 3D, and organoids that are developed from both 'normal' and tumor cells derived from patients[3].

In oncology drug discovery, *in vitro* models are traditionally used for experimental tumor modeling to identify lead compounds through high-throughput screening (HTS), evaluate potency and efficacy, understand mechanisms of action and target engagement, and fundamentally progress the development of new therapies.

But choosing the right *in vitro* model can be difficult. Each model comes with its own unique benefits and limitations, based on which one can understand when to apply them, respectively.

"Cell-based models are essential tools in basic scientific research and drug discovery. Thanks to the rapid progress in stem cell research and the pressing need for more clinically-relevant models, patient-derived organoids are being increasingly used and have revolutionized the landscape of recent cell-based drug discovery."

- Henry Li. Chief Scientific Officer at CrownBio -

3. Discussing Traditional 2D Cell Models

2D models have been applied in biomedicine since the early 1900s, and they remain a valuable tool in preclinical drug discovery and basic research. Grown in suspension or as an adherent monolayer, 2D cell cultures can be used in numerous applications throughout cancer drug discovery and development[4,5].

They have been extensively used in basic as well as applied research to understand signaling pathways, to study cell-cell interactions, to support and replace animal experiments, to probe the efficacy and safety of a compound, to investigate target engagement, and, to some extent, for biomarker discovery.

"*2D cell cultures are used to improve our understanding of basic cell biology and mechanisms of disease,"* Kevin Xu, Director of *In Vitro* Oncology at CrownBio said. *"In drug development, 2D cell lines are used to screen very large libraries of compounds in a high-throughput fashion. Due to their ease of use and rich historical data, scientists can also investigate mechanisms of drug action or changes in cell signaling following treatment.*"

In fact, 2D cell models have formed the foundation of our current knowledge of cancer biology and have been the basis of many ground-breaking discoveries.

Due to their long-standing adoption, 2D cell models come with a wealth of historical data, which has also enabled the development of standardized functional tests[4]. Stored in large commercial cell banks or storage centers, 2D cancer cell lines can be accessed easily. They are relatively simple to maintain, can produce quick results, and have lower maintenance costs than other cell models.

Despite these advantages, translating results acquired with 2D cell models to patients has been challenging[5]. While showing efficacy in 2D culture, some models are proven to inadequately predict drug response and give rise to a large number of possible targets that are often ineffective in clinical trials[5].

Moreover, although 2D cell models can be used to recapitulate disease pathways as observed *in vivo*, the actual cell-cell and cell-extracellular matrix interactions are not represented as observed *in vivo*[4,5]. Therefore, they cannot mimic the natural structure of cancerous tissue or tumors. This means that cancer researchers cannot study the effects of a compound on the tumor microenvironment, for example[4].

In addition, Jean-Pierre Gillet et al. discovered in 2011 that cancer cell lines – grown *in vitro* and *in vivo* – regardless of their tissue of origin, resemble each other more than the clinical samples they should be modeling[6]. This has been attributed to maintaining the cell lines in culture over a long period of time, in an artificial environment that bears little resemblance to an *in vivo* tumor. This, write the authors, emphasizes the need for new, more translatable *in vitro* cancer models[6].

The understanding that there had to be a change in preclinical *in vitro* models used in cancer drug discovery and development, soon spread throughout the global scientific community.

In early 2016, for instance, the US National Cancer Institute decided to discontinue its panel of 60 human cancer cell lines from its drug screening program in favor of more translational models[7]. This panel, NCI-60, was used by researchers around the world for more than 25 years and had been used to screen more than 100,000 compounds[7].

So while 2D cell lines remain valuable tools in basic cancer research, the need for more translatability and physiological relevant responses gave rise to the development of 3D cell models (See table, page 13).

4. Moving from 2D to 3D Cell Models

In their natural environment within a tissue, cells grow in 3D and establish cell-cell and cell-extracellular matrix interactions that are important to determine cell viability, influence cell signaling, and have an effect on drug response.

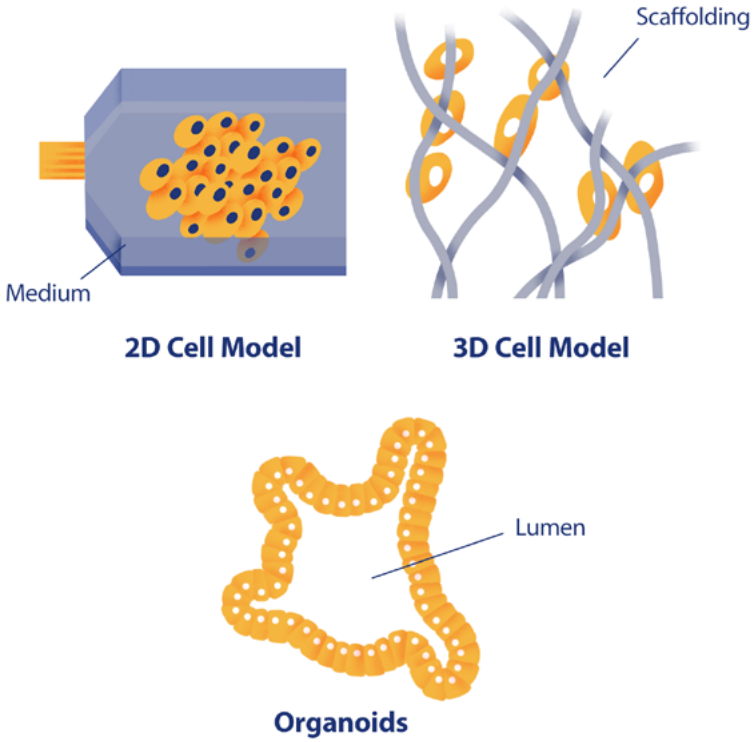
To better mimic the architecture of tissues *in vivo* and enhance the translatability of *in vitro* models, various 3D cell culture systems have been developed. These allow the growth of cell lines in a more physiologically relevant environment instead of on a flat plastic surface like 2D models. The resulting 3D structures are known as spheroids and have been used over the past few decades to study cell biology and to test drug response.

"*In 3D cell culture experiments, cells form physiological interactions with their surroundings, including with other cells and the extracellular matrix, which allow them to provide more physiologically relevant responses to stimuli and result in more accurate data on cell signaling, proliferation and importantly drug response,*" Kevin Xu explained.

Additionally, patient-derived models such as primary tissue have been have been cultured *ex vivo* in 3D with some success. These are sometimes referred to as histocultures. Compared to spheroids these systems better preserve the original tissue or tumor genetic make-up, thanks to never being adapted to grow on plastic, and therefore provide more patient-relevant drug responses.

However, original patient material can be challenging to source, and the success rate in establishing a primary cell culture in 3D is not the same across patients or tissue types. The life span of these primary models is also limited, as cells undergo a process called senescence, in which an irreversible cell cycle arrest mechanism occurs.

As a result, researchers may not have sufficient tissue banked for large scale pharmacology studies or may need to rederive the culture from banked material for repeat studies, resulting in greater variability of data. Compared to spheroids, therefore, these patient-derived systems have lower throughput and limited applications in drug testing and development.



Source: Adapted from Kapałczyńska, M. et al. 2018

Organoids represent the latest frontier in 3D tissue and disease modeling. They are derived from embryonic or adult pluripotent stem cells or from adult stem cells within epithelial tissues. Due to their stem cell origin, organoids have self-organizing and self-assembling properties that result in the development of a functional "mini-organ in a dish." These "mini-organs" preserve the multilineage identity of the original tissue *in vivo* as well as its genetic make-up and physiological functions, resulting in highly patient-relevant models.

Tumor organoids are derived from adult stem cells within a small patient-tumor fragment with similar pathophysiology as seen *in vivo*. Several published reports have demonstrated the high predictive power of patient-derived tumor organoids when compared to clinical response[8].

5. Understanding the Value of Organoids

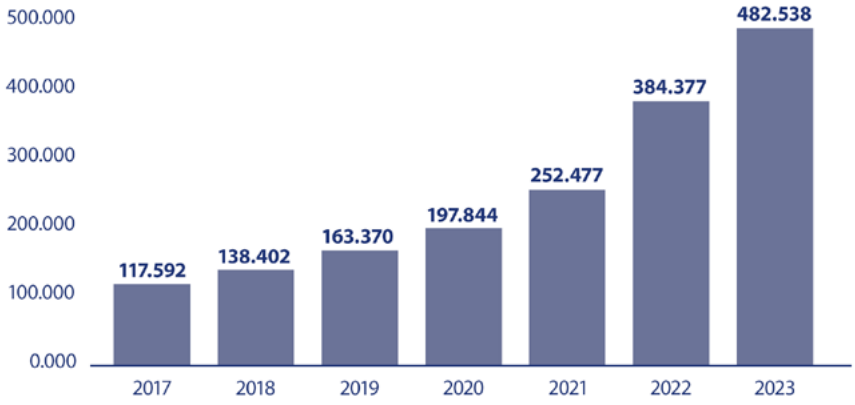
"Although the number of treatments available to cancer patients is increasing, it remains difficult to predict how individual patients will react to different treatments. The development of patient-derived organoids mimicking the biological features of patient tumors has allowed us to move clinical trials into the lab, in a dish. With that, we are making faster and more accurate predictions of patient response in the clinic."

- Sylvia Boj, Chief Research Officer at HUB -

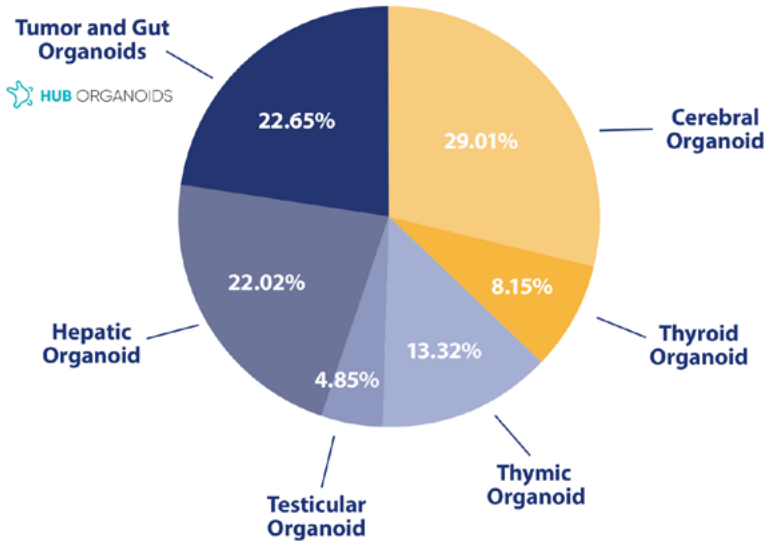
The huge potential of organoids is increasingly recognized in the global life sciences industry, and researchers are moving to adopt organoids for all stages of oncology drug development. In 2019, organoids reached a market value of $32M (€28M)[10]. With an expected compound annual growth rate (CAGR) of 28.11%, their market size is estimated to reach approximately $483M (€408M) by 2024[11].

Of the entire global organoid technology market share, tumor and gut organoids represent 22.65%[11]. The tumor and gut organoid segment is projected to reach a market size of approximately $113M (€95M) by 2023, growing at a projected CAGR of 27.15%[11]. Adult-stem cell-derived organoids (HUB Organoids™) represent the vast majority of the gut and tumor segment.

GLOBAL ORGANOID TECHNOLOGY MARKET FORECAST, 2017 TO 2023, US$ MILLION



Source: Knowledge Sourcing Intelligence Analysis

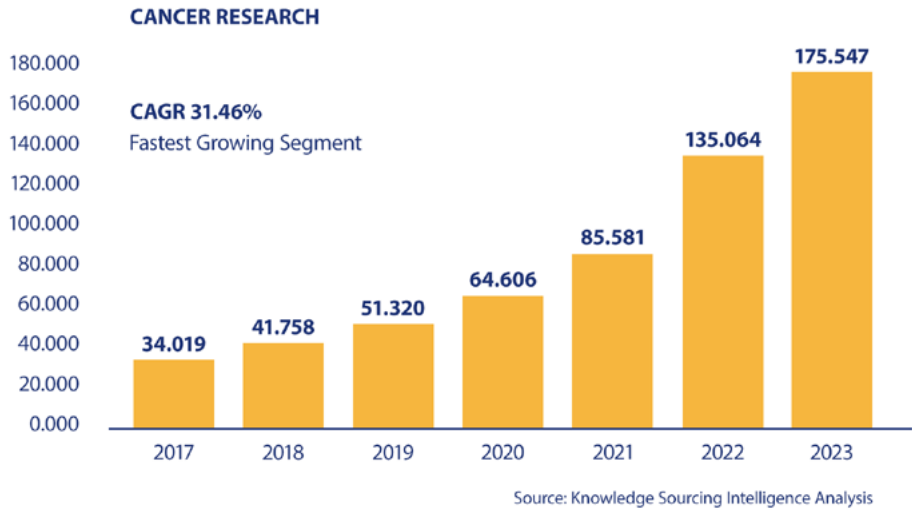


Source: Knowledge Sourcing Intelligence Analysis

Major investments into research and development to advance organoid technology is fueling the growth of the market[11]. Global advances in stem cell research and regenerative medicine are further boosting the development of organoid technology[11].

As organoid technology opens new possibilities for the development of more physiologically relevant cancer models, cancer research remains one of the fastest-growing segments on the market. It is closely followed by personalized medicine, which can benefit from organoids through rapid results, customized effects, and lower risks regarding adverse effects.

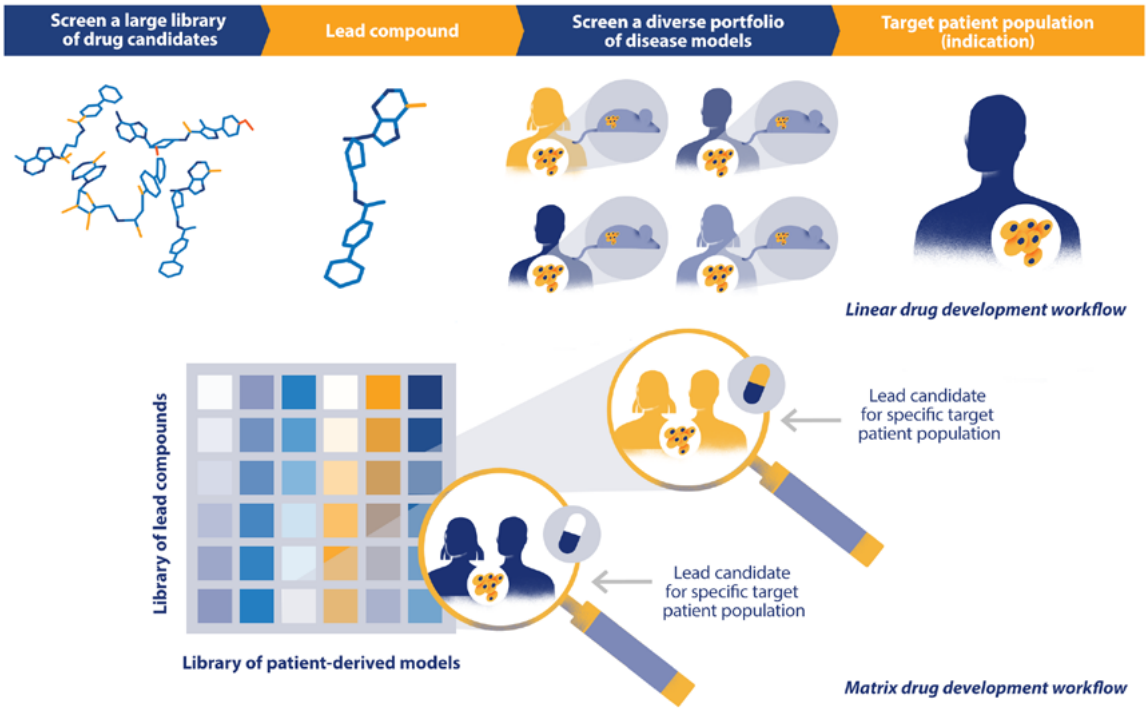
The trend will continue towards developing models that recapitulate the 3D architecture of original tumors, cell-cell interactions, and tumor-tumor microenvironment interactions[12].



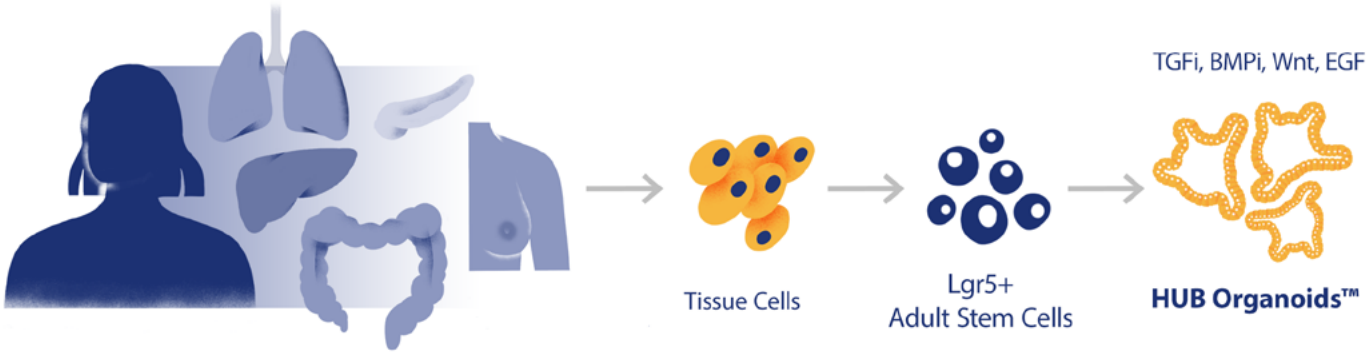
While the drug development industry gradually recognizes the need to adopt more clinically relevant models such as organoids to predict patient response, infrastructures and capabilities need to be built to enable the day-to-day use of organoids in drug development. This includes:

- A large and diverse tumor organoid model collection to reflect the patient population diversity, including several models for each cancer type, multiple cancer indications, mutational, and pharmacological profiles in a large biobank. This would allow researchers to study different responses across various models simultaneously, as well as identify biomarkers of response[13]
- High-throughput screening facilities to test multiple models, drugs, and drug combinations concurrently[13]
- Supportive bioinformatics capabilities in order to process the vast amount of data acquired
- Robust assays with efficient readouts. Establishing a number of robust assays and readouts to evaluate a compound's anticancer activity will provide trust and confidence in the generated data[13]

"This will make it possible to move away from a linear drug discovery and development process where an anti-cancer compound library is first screened to select a pool of lead agents that are then tested in preclinical models to find the most appropriate cancer indication to target," explained Henry Li. *"Instead, high-throughput screens can be performed using clinically relevant* in vitro *tumor organoids to select lead compounds and target indications simultaneously in a matrix discovery process."*



5.1. HUB Organoid Technology



Development of HUB Organoids

While organoids can be derived both from pluripotent stem cells (iPSC) and adult stem cells (ASC), epithelial cancer research greatly benefits from ASC-derived organoids. Although the existence of ASCs was widely accepted, it wasn't until 2007 that Nick Barker and his colleagues of the Hans Clevers group at the University of Utrecht discovered LGR5 as a stem cell marker in the small intestine and colon[14]. Following this discovery, researchers were able to identify ASCs in other epithelial organs, such as the stomach, liver, and pancreas.

In 2009, Toshiro Sato and colleagues at Clevers' lab showed that a single intestinal LGR5 stem cell could be used to grow self-organizing, multicellular structures they called crypt-villus organoids[15]. Two years later, Sato and his team adapted the culture conditions to enable the stable and long-term growth of mouse colon-derived and human small intestine- and colon-derived organoids in long-term culture[16].

These initial discoveries led to the development of an IP protected technology for the development of organoid models from nearly every adult epithelial organ and their corresponding carcinomas, as well as the foundation of Hubrecht Organoid Technology (HUB) whose mission is to foster organoid adoption among the scientific community.

Compared to iPSC-derived organoids, ASC-derived organoids (also known as HUB Organoids) don´t require a mesenchymal cellular niche to support stem cell growth and differentiation, which results in more rapid model development and a more stable culture over time. Most importantly HUB Organoid Technology is the only technology available for the development of tumor organoids directly from patient tumor biopsies.

Key Advantages of HUB Organoids

- Improved translatability of 3D *in vitro* data with clinically relevant organoid models
- Only available technology to develop tumor organoids for oncology drug development
- Faster development and enhanced scalability compared to other organoid technologies, patient-derived *in vitro* systems, or *in vivo* translational oncology platforms
- Living biobanks available to model patient population diversity and screen multiple agents and combination strategies concurrently
- Evaluation of *in vitro* drug potency and efficacy as well as off-target effects using tumor and healthy organoids

In an independently conducted study, Georgios Vlachogiannis and colleagues demonstrated the high clinical relevance of HUB Organoids by generating a live organoid biobank using tissue from patients with metastatic gastrointestinal cancer[8]. Importantly, when tested for drug response, the organoid biobank modeled the patients' response to treatment in the clinic. For the first time, the predictive power of the technology was demonstrated with 90% of patient-derived organoids responding similarly to the patients in the clinic.

"Tumor organoids represent an excellent and nearly physiological in vitro *tumor model system,"* said Xiaoxi Xu, Associate Director Cancer Biology and Immunology, who led the development of the organoid platform at CrownBio. *"As patient-derived models, tumor organoids recapitulate the pathophysiology of the original tumor and faithfully predict patient's drug response* in vitro. *In other words, tumor organoids have a greater translatability into humans and are more predictive than any other preclinical* in vitro *cancer model."*

5.2. Key applications of tumor organoids & what you need to exploit them

5.2.1. Organoids for high-throughput drug screening

In cancer drug discovery and development, tumor organoids are used to...

- Study drug responses
- Screen compound libraries
- Select the right *in vivo* model
- Identify predictive biomarkers
- Investigate mechanisms of action
- Assess immunotherapies and other treatments

One of the key advantages of patient-derived organoids and tumor organoids is that they can be kept in large live biobanks in which each organoid represents and maintains the genetic and phenotypic characteristics of its parental patient tumor[17].

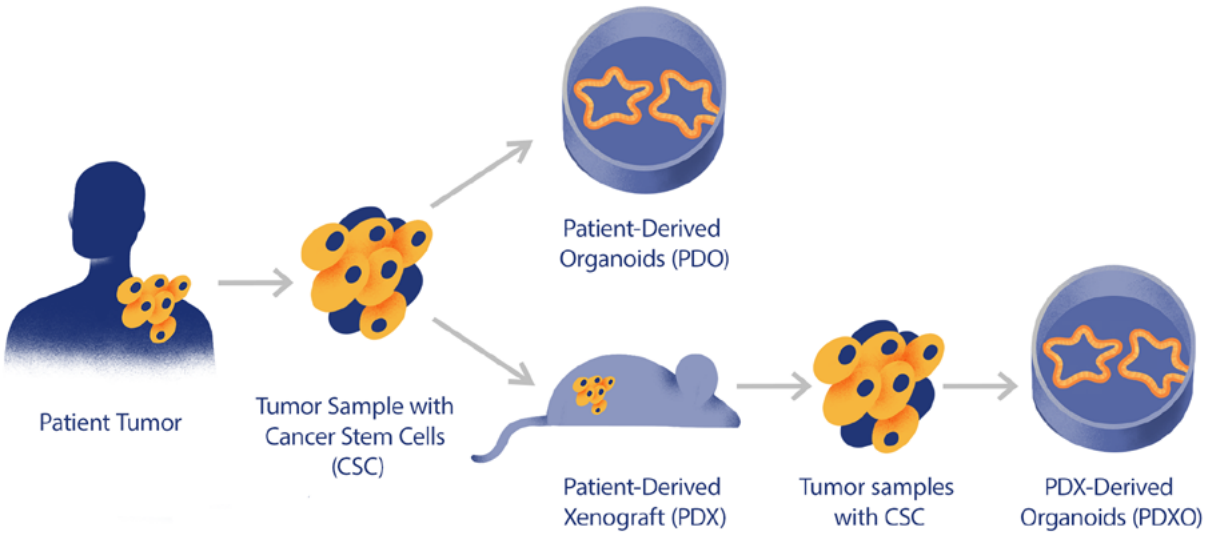
These features enable the use of patient-derived tumor organoids for high-throughput drug screening to determine gene-drug relationships and to support studies with other preclinical models, such as *in vivo* xenografts[17,18].

"Organoids have great scalability, which makes them adaptable to large screens testing multiple patients, candidate compounds, or combination strategies. This helps companies fast track drug efficacy evaluation" explained Xiaoxi Xu.

5.2.2. Matched organoids and *in vivo* models

By matching *in vitro* organoid models with *in vivo* models, researchers have the unique opportunity to transition from *in vitro* to *in vivo* and validate response data and predictive biomarkers[19].

In 2019, CrownBio partnered with HUB to access their exclusive HUB Organoid Technology and develop the largest commercial most diverse commercial biobank of tumor organoids for oncology drug development.



Development Process of Patient-Derived & PDX-Derived Organoids

Thanks to its model development expertise, CrownBio has established an extensive collection of matched *in vivo* PDX and *in vitro* PDX-derived organoids (PDXO), which have been shown to share key genomics and transcriptomics features as well as drug response.

"*At CrownBio, our expertise in model development allowed us to leverage our established collection of PDX models to generate new organoid models, PDXO. We understood that there was a need for additional cancer indications, mutational, and pharmacological profiles than currently available*" described Li.

These newly developed PDXO models expand the repertoire of currently available patient-derived organoid (PDO) models to more than 15 different cancer types and various pharmacological and mutational profiles. This provides the opportunity to represent the diversity of the patient population when conducting *in vitro* screens to investigate drug response and identify predictive biomarkers.

"*Living biobanks of patient-derived organoids can be established to capture the diversity and heterogeneity of patients and used in population studies to identify responders and non-responders. By using clinically relevant organoid models and matching them with* in vivo *models, companies can obtain more predictive drug efficacy data before they reach clinical trials and therefore have a better chance of success,*" Xiaoxi Xu summarized.

Moreover, new *in vivo* xenograft models can be developed using existing organoids therefore providing new matched pairs to test hypotheses and to study an agent's effects in a more complex system.

5.2.3. Genetic manipulation of tumor organoids

Organoids, much like immortalized cell lines, can be engineered via gene transfer, gene editing, or RNA interference to study the contribution of different genes in cancer development[20].

Organoids can be engineered to introduce reporter genes such as luciferase. Patient-tumor labeling to generate preclinical *in vivo* models for research is practically impossible to obtain otherwise. This provides an unprecedented opportunity for labeling tumors and generating new orthotopic xenograft models where disease progression and response to treatments can be tracked in real-time via optical imaging.

6. Choosing the Right Cell Model for Your Cancer Research

"*To better translate promising preclinical drug candidates into clinical success, a biotech may need to think carefully about its cell model choices,*" Kevin Xu emphasized. "*It is generally advisable to adopt at least one advanced cell culture system in addition to the traditional 2D cell culture models. Alternatively, a biotech may want to adopt an advanced cell-based model right from the beginning, and this would also require some consideration on the optimal model type.*"

For instance, 2D cell lines are quick and relatively inexpensive tools for basic research, to investigate signal transduction pathways, a drug's mechanisms of action, and target engagement, whereas patient-derived models are better suited for predicting drug responses and understanding disease mechanisms.

However, conventional patient-derived *in vitro* models remain difficult to scale up, which is where organoids come in. In contrast, organoids can be more easily scaled up, recapitulate the 3D architecture of the parental tissue, and have shown high predictive power of clinical response.

HUB Organoids are only available for epithelial tissues and their carcinoma, therefore there still remains a need for other advanced model types to study non-epithelial tumors and their drug response.

In short, selecting the right preclinical *in vitro* models throughout cancer drug discovery is a critical step to increase the chances of success in a drug development program. More and more drug developers are partnering with experienced providers in the preclinical space to help guide their model choice.

When choosing a preclinical provider, it is important to consider the breadth and depth of their model collection, which should include matched *in vitro*/*in vivo* models for an informed transition.

Population studies are increasingly popular among drug developers too, particularly in the form of matrix high-throughput screening where a lead candidate and a target patient population can be selected at the same time to accelerate and improve efficiency in drug development.

Advantages and limitations of 2D cell lines, conventional 3D cell models, and organoids

	2D Cell Lines	Conventional 3D Models (Spheroids, Histocultures, and *Ex Vivo* Systems)	HUB Organoids™
Benefits	Good culture quality: high performance, reproducible, long-term, easy to interpret, and simple to establish A large collection of models available Low costs Widely commercially available tests Rich historical data for reference Amenable to engineering	More closely mimic the tissue and tumor architecture observed *in vivo* compared to 2D systems Greater physiological relevance than 2D models thanks to mimicking physiological conditions, such as hypoxia Enhanced translatability to *in vivo* compared to 2D models Suitable for use with primary tissue in short term culture	Good culture quality: stable, long-term culture, highly reproducible, and suitable for primary tissue Highly predictable and translatable into humans compared to 2D and other 3D models Cost-efficient thanks to minimizing the need for repeat studies by producing high quality and more predictive data Matched *in vivo* models available
Limitations	Poor translatability *in vivo* as they don't mimic the natural tumor or tissue structure	Unstable in long-term culture due to cellular senescence which hampers model scalability and can affect data reproducibility Translatability to *in vivo* still not ideal Some restriction on engineering depending on model type Limited tissue availability challenges scalability	More complex to establish than 2D models Some cancer indications are particularly challenging to establish as organoids Only available for epithelial organs/ carcinomas
Applications to Oncology Drug Development	2D cell lines form the basis of our understanding of cancer biology and are valuable to investigate target engagement and dissect signaling pathways at a large-scale, quick, and cost-effective. HTS – lead identification Workhorse models but limited use for clinical hypotheses testing	Investigate disease mechanisms and drug response in a more patient-relevant system compared to 2D cell lines. Translational studies when primary tissue is used Investigate drug penetrance and the contribution of stromal elements in co-cultures	Organoids are clinically relevant, more rapidly developed compared to *in vivo* systems, and more easily scalable than other patient-derived *in vitro* models. These features make them particularly adaptable to large scale HTS of candidate compounds as well as population-based studies or "clinical trial in a dish" approaches Provide an opportunity to revolutionize drug development by shifting linear processes to a matrix, high throughput system Matched *in vivo* models available for continuity of biology from *in vitro* to *in vivo* testing or more refined decision making on which model to select for *in vivo* as well as which drug/combination Can be co-cultured with stromal elements

7. Discovering CrownBio's Cell-Based Models

As a company with a longstanding experience in preclinical oncology drug discovery, CrownBio recognized that the high failure rates for anticancer agents need to be tackled with a more informed model choice at each stage of drug development and more translatable preclinical models to better predict drug response in the clinic.

To progress and improve early and translational stages in *in vitro* drug development, CrownBio is committed to providing an array of models and services from 2D cell lines, 3D/*ex vivo* models, and organoids used as standalone, in parallel, or in a sequential workflow.

Apart from offering an extensive portfolio of well-characterized 2D cell lines for the earliest stage of drug development, the company is also building the largest commercial patient-derived tumor organoid library available today in partnership with Hubrecht Organoid Technology.

To complement and expand the HUB Organoid™ biobank CrownBio has developed a large library of PDXO models with matched *in vivo* PDX for informed *in vitro* to *in vivo* transition.

CrownBio's large and diverse collection of tumor organoids reflects the diversity of the patient population and can be used in large-scale, high-throughput drug screens to help select the most promising candidates, as well as identify suitable patient populations[21].

Moreover, for immunotherapy studies, CrownBio has developed an organoid and immune cell co-culture platform that can be used to evaluate the potency of immunotherapies, assess tumor organoid killing by engineered T cells, determine tumor reactivity of CAR-T and TCR T cells, as well as identify antigens of interest and profile immunotherapy target gene expression[22].

CrownBio's expertise in the use of 2D cell models includes well-established 2D cell-based assays using panels of extensively characterized cancer cell lines with genomic, gene expression data, mutational profiles, and IC_{50} data available via the online database XenoBase® for over 700 cell lines. The company offers a variety of *in vitro* platforms for oncology drug discovery, including efficient screening programs such as OmniScreen™.

OmniScreen is CrownBio´s large-scale screening platform of over 500 well-characterized cancer cell lines for testing single agents or combination strategies. The platform supports early-stage decisions on candidate lead selection by offering data on tumor-killing activity, and upon bioinformatics analysis, novel targets can be identified and hypothesis generated.

CrownBio´s cell lines can also be run in a 3D format as spheroids for enhanced physiological relevance from customer´s drug screening.

Leveraging its extensive collection of over 3000 PDX models, CrownBio has developed an *ex vivo* platform consisting of more than 200 PDX tumor-derived cell lines grown in 3D (3D *ex vivo* PDX) to perform 3D clonogenic assays and assess cell viability following drug treatment. The 3D *ex vivo* PDX includes unique models with patient-relevant mutations involved in drug resistance as well as subpanels of brain, blood, and kidney cancer that are not available using alternative 3D technology.

Finally, CrownBio is able to offer PrimePanel™, a collection of a collection of PDX derived cell lines to assess drug treatment in low passage models directly derived from tumor cells.

"*Our mission is to offer a revolutionary workflow to cancer drug discovery by incorporating the most translatable 2D and 3D* in vitro *models available into oncology drug development programs as well as matched patient-relevant in vitro and* in vivo *systems for optimal predictivity,*" Henry Li concluded.

3D cell line models, including revolutionary organoids, offer a promising new avenue for the development of effective anticancer treatments. Data from these advanced models coupled with early-stage investigations in 2D cell lines will result in greater confidence from preclinical data and will accelerate the development of new, more effective drugs.

8. Appendix

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