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Lung organoids © Andreas Hocke

Human
3D ORGAN MODELS
NETWORKING EVENT

ABSTRACTS

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1

3D Bioprinting of Humanized Xeno-free Liver Model

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Although significant efforts are being made to develop new approaches to replace animal testing, they frequently involve the use of animal-derived components. The present study aims at developing a bioprinted liver model that is completely devoid of animal components. Huh-7 cells were adapted to a chemically defined medium using both direct and sequential approaches, resulting in cells with a relative viability of 80-90% and similar behavior to cells cultured in 10% FCS. Three different non-animal freezing solutions were also prepared for the cryopreservation of these cells. A bioink was formulated based on sodium alginate, human collagen (I), and nutrient supplements to avoid the use of animal components in bioprinting. The resulting xeno-free bioprinted liver models demonstrated high cell viability and metabolic activity, comparable to that of the Matrigel-based liver model. To assess the applicability of this xeno-free model, it was used to test the hepatotoxicity of okadaic acid, with results compared to conventional 2D cell culture. The study findings suggest that the xeno-free 3D bioprinted liver model shows great potential as an alternative to animal testing for hepatotoxicity evaluation.

2

Culture medium-dependent effects of ivacaftor on the rescue of F508del-CFTR in primary nasal epithelial cultures from patients with cystic fibrosis

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Background

The introduction of highly effective CFTR modulator therapy with elexacaftor/tezacaftor/ivacaftor (ETI) led to a substantial clinical benefit in patients with cystic fibrosis (CF). In vitro studies suggest that potentiator compound ivacaftor as part of ETI triple combination therapy may impair correction efficiency of F508del-CFTR. However, quantitative studies of this unwanted effect on CFTR rescue and its impact on mucociliary transport in primary human airway epithelial cultures remain limited.

Methods

Primary nasal epithelial cells were collected from 13 patients homozygous for F508del-CFTR by nasal brushings and cultured under air-liquid interface (ALI) conditions using two common differentiation media: UNC medium (in-house made according to Gentsch et al., 2017), or PneumaCult medium. Differentiated cultures were pre-treated with (i) elexacaftor/tezacaftor (ET); (ii) ETI; or (iii) DMSO (vehicle control) for 48 hours. Rescue of CFTR chloride channel function was assessed by transepithelial short-circuit current (Isc) measurements in Ussing chambers and effects on mucociliary transport rates (MCT) were assessed by videomicroscopy after addition of fluorescently labeled particles.

Results

CFTR-mediated currents, as measured by forskolin-induced and CFTRinh172-sensitive Isc, were significantly increased in both culture media by ET or ETI treatment compared to DMSO. In UNC medium, chronic presence of ivacaftor had no effect on amiloride-insensitive Isc, but reduced forskolin-induced ($\Delta\text{Isc}_{\text{ET}} = 1.57 \mu\text{A}/\text{cm}^2$; $\Delta\text{Isc}_{\text{ETI}} = 1.02 \mu\text{A}/\text{cm}^2$, $p < 0.05$) and CFTRinh172-sensitive Isc ($\Delta\text{Isc}_{\text{ET}} = -2.00 \mu\text{A}/\text{cm}^2$; $\Delta\text{Isc}_{\text{ETI}} = -1.35 \mu\text{A}/\text{cm}^2$, $p < 0.01$). In PneumaCult medium, chronic presence of ivacaftor increased amiloride-insensitive Isc, ($\Delta\text{Isc}_{\text{ET}} = 0.11 \mu\text{A}/\text{cm}^2$; $\text{Isc}_{\text{ETI}} = 2.36 \mu\text{A}/\text{cm}^2$, $p < 0.01$) and decreased forskolin-induced Isc ($\Delta\text{Isc}_{\text{ET}} = 7.32 \mu\text{A}/\text{cm}^2$; $\Delta\text{Isc}_{\text{ETI}} = 5.69 \mu\text{A}/\text{cm}^2$, $p < 0.01$) and similar CFTR inh172-sensitive Isc compared to ET. Furthermore, ETI-treated PneumaCult cultures displayed enhanced MCT compared to ET and DMSO-treated cultures.

Conclusion

These data show that differences in ALI culture medium composition affect functional F508del-CFTR rescue in vitro. Increased amiloride-insensitive Isc and increased MCT in ETI- vs. ET-treated PneumaCult cultures suggest that the presence of ivacaftor is important for constitutive CFTR activation and improvement of mucociliary clearance.

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3

How to image your organ model – ExPerimental Imaging at the Charité to support 3R (EPIC3R)

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Biomedical imaging, such as magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), intravital optical imaging (OI) or computed tomography (CT) allow noninvasive morphological and functional investigations in living systems. These technologies are also an important tool for translational research since most clinical monitoring of disease and therapy heavily relies on imaging and methods developed in smaller model systems can often be scaled to human dimensions. However, most researchers working with cell cultures and organoid models primarily rely on microscopy and are not aware of the capabilities of biomedical imaging.

EPIC3R is an umbrella of all preclinical imaging labs at the Charité. It is also closely connected to microscopy facilities. We present preliminary results on biomedical imaging of alternative cell culture models, e.g. of vascularization of kidney organoids or tumor cells transplanted on the chorioallantoic membrane (CAM) of embryonated chicken eggs. Most importantly, we are now aiming to further expand this research – together with you!

We will present the available imaging modalities and our vision of how we can help 3D organ model researchers using the excellent infrastructure readily available at the Charité.

4

Modeling the tumor microenvironment by establishing organotypic 3D model systems

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The close interaction of tumor cells with their tumor microenvironment (TME), a diversified ecosystem of non-malignant host cellular and acellular components, plays an important role in generating tumor heterogeneity and enhancing multi-drug resistance. Hence, establishing organotypic cancer models modeling the complexity of the tumor and its surrounding may serve as a powerful platform to facilitate efforts in precision oncology.

Three different 3D model systems from patients with breast cancer (BC) and colorectal cancer (CRC) will be established and characterized, including organoids (PDOs), air-liquid interface cultures (ALI-PDOs) and multicellular spheroids. Over a time span of two months nine tumor samples were obtained (8/9 BC, 1/9 CRC), resulting in an initial take rate of 88,9% for PDOs and 77,8% for ALI-PDOs. By performing immunofluorescence (IF) cell stainings in several passages and multi-omics approaches, the potential of the organotypic models to preserve the TME and molecular characteristics of the original tumor is analyzed. Additionally, comparative drug testings are planned to be performed in order to evaluate the functional characteristics of the established cancer models. To address the clinical practicability, we aim for optimizing the preclinical drug testing procedure to reduce the time span between sample collection and the report of drug sensitivity.

By implementing the established and characterized organotypic models into clinical trials within the upcoming *National Center for Tumor Diseases* (NCT), their predictive power for model-based guidance of treatment decision-making as well as their clinical application for specific therapeutic settings (e.g., immunotherapy) will be evaluated.

5

Towards a human iPSC-derived neuronal network for high-throughput cognitive drug screening

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hiPSC-derived neuronal cell culture has been an emerging field for about a decade. Fast neural fate induction through NGN2 expression has allowed labs without a focus on stem cells to implement stem cell-derived human neurons in their cell culture, with the prospect of a better translation compared to mouse-derived systems as well as a reduction of the number of experimental animals. hiPSC-derived neuronal networks are promising for cognitive drug discovery to treat neurodegenerative diseases, but this requires a network capable of synapse strengthening and weakening.

So far, the field has been able to show that human induced neurons (iNeurons) cultured for a sufficiently long time (variable depending on the differentiation protocol) express pre-synaptic markers and show activity in multi-electrode recordings. But convincing post-synaptic immunostainings are rare, as are functional assays investigating pre- and post-synaptic function and synaptic maturation.

With our differentiation and maturation protocol, iNeurons begin to show synaptic expression of PSD95 at DIV 45, but not at DIV36. DIV 45 iNeurons are further capable of taking up and releasing FM1-43 dye, a fluorescent styryl dye that inserts into the membrane and is taken up through synaptic vesicle recycling, leading to a punctate fluorescent synaptic signal. The dye release kinetics follow an exponential decay indicating mature synaptic vesicle recycling.

To examine post-synaptic development and function, we will stain iNeurons at different DIVs with phalloidin, which stains actin filaments and can be used to visualise dendritic spines, where mushroom spines correspond to mature post-synaptic sites. We will further study post-synaptic receptor function through receptor localisation and recycling assays.

By characterizing when and to what extent iNeurons develop mature synapses, we aim to establish a standardized hiPSC-derived neuronal culture system that is capable of synapse strengthening and weakening and can be used for high-throughput screening of cognitive drugs.

6

An autologous model of the adaptive immune response in human lung organoids: Targeting infection with influenza-specific T-cells

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Zoonotic respiratory viruses contribute to substantial mortality in humans and represent the main drivers of disease pandemics. Severe viral infections cannot be treated sufficiently with anti-viral drugs and there is an unmet need for novel therapeutics that steer a protective anti-viral T cell response. Patient-derived anti-viral T cells represent a promising alternative in the fight against infections. However, these T cell therapeutics require a suitable pre-clinical, human based evaluation platform replacing animal models that poorly reflect the human pathogenesis.

In our project, we are going to shed light on the advantages of Influenza-A (IAV) specific anti-viral T cell products (TCPs) as therapeutic option. This will be achieved by the establishment of a novel autologous platform using IAV infected lung-organoids (iPSC/adult stem cell derived (ASC)) and IAV specific TCPs. The specific elimination of infected lung epithelial cells and protective features against IAV related infection will be highlighted by high-end 4D imaging, determination of viral load, single cell omics or immune profiling. We have shown that human lung organoids derived from ASCs can be used as infection model for IAV. Furthermore, infection was improved by the induction of apical-out conformation allowing for a more physiological interaction between T cells and infected epithelium. In addition, we have successfully generated iPSC-derived human lung organoids and confirmed IAV infection upon apical-out inversion as a proof-of-concept approach. We are confident in manufacturing IAV-reactive TCPs and have proven their IAV-specific properties. At this stage, we are establishing co-culture conditions for both cell models focusing on e.g., antigen-presentation. This human based, autologous infection model will help to bypass extensive animal usage and provide relevant insights into safety and efficacy of IAV specific TCPs. Finally, this platform will enable pre-clinical and animal-free evaluation of a broad range of adoptive T cell therapeutics with diverse specificities for clinical use.

7

Animal-free *in vitro* 3D synovial membrane model mimicking the pathogenesis of arthritis

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Osteoarthritis (OA) is the most common joint disease, with rheumatoid arthritis (RA) representing the most common autoimmune arthritis. Fibroblast-like synoviocytes (FLSs) of the synovium are critical drivers of arthritis. In contrast, macrophage-like synoviocytes (MLSs) have been implicated in maintaining barrier function. However, the impact of FLSs and their interaction with MLSs on cartilage degradation remains elusive. Here, we aimed to develop an animal-free, human-centered, *in vitro* 3D synovial membrane model that reflects the (patho-)physiology of the synovium to study the course of arthritis and new therapeutic strategies.

Bone marrow-derived mesenchymal stromal cells (MSCs) were used to reflect the physiological state compared to FLSs from OA patients. Blood-derived monocytes were differentiated into either M1 or M2 macrophages, with M2 mimicking MLSs. Cells mimicking FLSs and MLSs were incorporated into a synthetic RGD hydrogel and stacked layer-by-layer, mimicking the lining and sublining layer in an animal-free *in vitro* 3D model. Tumor necrosis factor- α treatment increased, e.g., FLSs proliferation reflecting pannus formation typical of RA. Transforming growth factor-beta and OA synovial fluid induced an OA-like fibrotic phenotype as evidenced by increased expression of *ACTA2* (actin alpha-2, smooth muscle) and *SERPINE1* (serpin family E member 1) compared to the untreated control. M2-MLSs in the lining layer significantly attenuated both effects.

Our *in vitro* 3D synovial membrane model allows for studying cytokine-driven cellular changes and cell-cell interaction in a defined manner. Prospectively, we aim to provide an *in vitro* alternative for preclinical drug screening by incorporating both M1 and M2 macrophages.

8

Towards a „Head and Neck cancer atlas” – Comprehensive analysis of spatially distinct tumor samples

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Head and Neck squamous cell carcinoma (HNSCC) is the seventh most commonly diagnosed cancer worldwide[1]. Despite the efforts of approving targeted therapeutics as treatment options besides radiation and chemotherapy, the incidence and mortality of HNSCC patients are still stagnating over the past two decades. One of the reasons for such a low survival rate is the intratumoral heterogeneity (ITH) of HNSCC. To investigate HNSCC-ITH and its potential clinical significance, spatially distinct samples from the same tumor specimen were collected and processed for a comprehensive analysis by whole genome (WG) and whole transcriptome (WT) sequencing, single-cell RNA (sc-RNA) sequencing, histological analysis, and for the establishment of preclinical models. WGS analysis showed that 40% of the patients harbor clonal diversity, with so far unstudied clinical significance. Histochemical analysis of corresponding patient samples underlined the existing genetic ITH, as heterogeneous expression levels, particularly of PD-L1, p53, vimentin, and CAIX, were observed (paper submitted)[2]. 42 biopsies from 10 patients were collected and dissociated into single cells, hereby using 1/3 of each sample for the establishment of the spatially distinct preclinical models, resulting in a take rate of 33.3% (19.0% established from the tumor center, 14.3% established from the rim). Next, drug treatments will be performed based on the molecular and expression signature of corresponding tumor specimens and derived preclinical models to investigate whether organoid cultures can recapitulate HNSCC-ITH genetically and functionally. In-depth characterization of this unique sample set will allow us to identify further predictive biomarkers, and molecular and immunologic targets and to understand if analysis of spatially distinct tumor samples should in the future be incorporated into clinical routine since it may have an impact on patient stratification.

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9

Decoding the role of histone modifier mutations in human neurodevelopmental disorders (NDDs) with multi-OMICs analysis of (vascularized) brain organoids.

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Keywords: histone modifications, neurodevelopmental disorders, vascularized cortical organoids

The development of the human brain is an intricate process that relies on precise epigenetic regulation of transcription factor-gene networks to coordinate cell differentiation and tissue formation (Rothbart & Strahl, 2014). Histone modifying enzymes are key regulators of chromatin accessibility and the growing number of neurodevelopmental disorder (NDD)-associated mutations found in histone 3 lysine 4 (H3K4)-modifiers highlights their importance in CNS development. Mutations in the chromatin modifying demethylase LSD1 and the methyltransferases MLL1 and MLL2, have been linked to NDDs including developmental delay and intellectual disability (Vallianatos & Iwase, 2015).

In this work we hypothesize that the pathogenic mutations in LSD1, MLL1/2 are disrupting transcription factor-gene networks not only in neurons, but also in neural stem cells, glia and vascular endothelial cells, thereby indirectly impairing neurodevelopmental processes.

To test this hypothesis, we will use joint single-cell multi-OMICs approaches (ATAC-Seq/RNA-Seq) on a combination of 2D and 3D in vitro models of the human brain derived from healthy and LSD1, MLL1/2 mutant stem cell lines. Intriguingly, some NDD patients exhibit both neural and vascular pathologies, pointing toward a crucial role for H3K4-modifiers in regulating neurovascular codevelopment in the embryonic brain (Digilio et al., 2017; Yoon et al., 2015). By generating vascularized cortical organoids carrying the H3K4-modifier mutations, we aim to investigate neural or vascular lineage-specific effects that may underlie perturbed brain development in NDDs. Currently we are establishing cortical organoid formation protocols in our group, using a previously described approach for generating human cortical spheroids (hCSs) (Paşca et al., 2015).

Having established our model systems, we will characterize the chromatin and gene expression landscape of the neurogenic and neurovascular niche in normal human brain development and disease, explore how environmental factors can affect the pathology of the H3K4-modifiers and examine how glia cells contribute to the phenotype of the diseases.

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10

Optogenetic-based *DICER1* perturbation to study microRNAs function in human neurogenesis

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MicroRNAs (miRNAs) are small non-coding RNAs with crucial roles in cell-type specific gene regulation and particularly abundant in the mammalian brain. Few miRNAs were found in different animal models to coordinate cell differentiation during development. Across species, the speed of neurodevelopmental processes differs and so the timing where gene regulators like microRNAs are required.

Here we aim at investigating the role of microRNAs in controlling different stages of neurodevelopment – from early neurogenesis to cell maturation – in a complex human tissue context, recapitulated by human cerebral organoids. In order to knock-down microRNAs with spatiotemporal resolution, we generated a human induced pluripotent stem cell line where the *DICER1* locus is disrupted by a light-inducible Cre/Lox system. We optogenetically knocked-out *DICER1* at different stages of neurogenesis and assessed the molecular consequences at different time points in cerebral organoids.

In preliminary experiments, photo-stimulated organoids displayed recombination of the targeted locus and consequent knock-down of the *DICER1* mRNA and selected microRNAs. Neurogenesis was impaired over time.

With further experiments, we will assess the impact of the knock-out on global microRNAs production and on differentiation trajectories by employing small RNA sequencing and single-cell RNA sequencing. Next, we intend to investigate the role of microRNAs at later time points of organoid development (i.e. after neurogenesis) for proper cortex formation, with also the perspective to perform local knock-out inductions, targeting specific cell types (e.g. neural progenitor cells) and tracking their specification fate over time.

Establishment and characterization of patient-derived organoids from HNSCC for analyzing mechanisms of radioresistance

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Achieving personalization in treatment of head and neck squamous cell carcinomas (HNSCC) requires adequate models. The study aims at the establishment of patient-derived organoids (PDOs) from HNSCC, and the assessment of their potential value in biomarker discovery for radioresistance and screening for radiosensitizing agents

The ethics committee approved this study (EA1/152/10). Tumor tissue from patients with informed consent was collected during diagnostic or curative surgery at the maxillofacial and otorhinolaryngology departments at the Charité. After tissue dissociation and expansion in monolayer cultures, cells were seeded in Matrigel® to form organoids. Statistical analysis was performed with SPSS. Organoid sections were haematoxylin-eosin and immunohistochemically stained for p40, CK5/6 and Ki67. The *ex vivo* assessment of radiosensitivity was established using 3D cultures of radioresistant / -sensitive subclones from the FaDu cell line. The protocol was then applied to PDO models.

Overall efficiency of PDO generation from primary tumor specimen from HNSCC patients was 45%. Histopathological characterization confirmed their SCC-phenotype. The majority of models were from male patients (64%) and actual / former smokers (71%). Neither the tumor localization nor the sample type (biopsy vs. surgical specimen) was decisive for successful organoid generation. Samples from recurrent or persistent tumors after radiotherapy showed a significant lower engraftment rate (33.3%) compared to treatment naive specimens (80%) ($p < 0.001$). With our irradiation protocol, we were able to distinguish radiosensitive from resistant FaDu models. Preliminary results from PDOs ($n=3$) showed a dose-dependent decrease in proliferation, cell-viability (CellTiterGlo®) and clonogenic potential, and a considerable interpatient variability in radiosensitivity.

To our knowledge, this is the largest collection of HNSCC PDO models established so far. Multi-omics characterization is currently ongoing. PDOs might be valuable models to investigate individual responses to radiation therapy and the molecular mechanisms underlying radioresistance. In future studies, we will also focus on improving organoid engraftment from radioresistant tumors.

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Harnessing ASC-derived lung organoids for pneumonia-related infectious disease research

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In the aftermath of a respiratory disease pandemic, the need for rapid, reproducible, human lung models for risk assessment of emerging pathogens became evident. The human lung, however, comprises a large variety of cell types including diverse epithelial, interstitial, endothelial and immune cells, particularly alveolar macrophages (AMs). By deriving lung organoids from adult stem cells (ASCs), we not only aim to model the cellular composition but also the spatial arrangement of the human alveolar and bronchial compartment, creating a robust system to investigate pneumonia-related infectious diseases.

Single-cell RNA sequencing revealed that ASC-derived lung organoids express a variety of marker genes for different cell types, including basal, ciliated, secretory, and alveolar type 2 cells. Using our previously generated dataset, we searched for specific cell markers and validated their protein expression by multiplex imaging and semi-automated segmentation, revealing a detailed organoid cell composition. Genetic editing of organoids facilitates further research into specific protein functions. The pure epithelial identity of organoids compared to tissue samples herein provides a benefit to the understanding of specific cell types. Based on this, profound infection and microinjection experiments are carried out under the use of viral and bacterial pathogens. However, immune responses of the lung are highly dependent on AMs. Hence, the addition of AMs to organoids, either in suspension or in a hydrogel basement membrane extract, is an important first step towards immunocompetence of the model.

In characterizing and diversifying the cellular composition of ASC-derived lung organoids, we constantly evolve a robust human lung model system allowing for investigation of complex (patho-) physiological mechanisms beyond infection.

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Establishing prostate cancer organoids from radical prostatectomy specimen

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Background: To date, prostate cancer organoids from primary cancer tissue could only be established with low success rates. One of the biggest challenges is overgrowth and organoid formation of normal prostate cells.

Material and Methods: We were able to obtain ten targeted punch biopsy samples from eight patients with localized prostate cancer, which underwent radical prostatectomy. In a literature search we identified Prostate Specific Membrane Antigen (PSMA) and Prostate Stem Cell Antigen (PSCA) as promising cell surface markers for cell sorting of malignant cells before seeding organoids.

Results: We detected tumor tissue in four out of the ten samples. The average PSA value of the included patients was 14,7ng/ml. In the positive punch biopsies, one samples had an International Society of Urological Pathology (ISUP) Grade 2, two samples ISUP 3 and one sample ISUP 5. We did not find tumor in four out of the eight samples (negative samples), all these samples had an ISUP Grade 2.

Discussion: These are important initial steps to establish a robust protocol for growing prostate cancer organoids from primary tumor tissue. Our sampling approach seems to be more successful in ISUP Grade 3. Further steps include improving the sampling protocol, Fluorescence Activated Cell Sorting (FACS) of viable PSMA+/PSCA+ cells and testing different media and hypoxia conditions.

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Generation of stem cell-derived human hepatocytes with programmable functions

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Current hepatocyte differentiation protocols from induced pluripotent stem cells produce immature hepatocytes. The levels of expression of metabolic genes, such as CYP3A4 and UGT1A1 among others, are insufficient for the use of these hepatocytes in drug metabolism studies or clinical applications. As a proof-of-concept, we programmed the expression and function of two mature, clinically relevant liver-specific enzymes CYP3A4 and UGT1A1 that were not fully expressed using our conventional hepatocyte-directed differentiation protocol. Using an inducible dCas9-based activation system strategy, we were able to maintain the expression of hepatocyte-enriched transcription factors essential for hepatocyte function, induce expression of the clinically relevant enzymes CYP3A4 and UGT1A1, and most importantly, demonstrate metabolic activity at levels comparable to or greater than those observed in freshly isolated primary human adult hepatocytes.

These human iPSCs-dCas9gain could be used to generate any cell type or tissue and control the expression of single or multiple genes. Consequently, the current method is a potent instrument that can be used to investigate signaling pathways and generate human disease models. In addition, applications of human iPSCs-dCas9gain in positive and negative selection screens will enable the analysis of numerous types of genetic components, including protein-coding genes, noncoding RNA elements, and epigenetic modifiers of diverse biological processes.

Future research will optimize these tools for disease modeling within structured microphysiology systems (MPS) and organoid-MPS, including diseases affecting the liver that are of critical importance. These studies outline a strategy for engineering liver tissue for potential future biological, drug discovery/development, and clinical applications.

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Assessing Alternatives to FCS and Different Media for the Culture of *ex vivo* Human Skin

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Our previous study has demonstrated that the *ex vivo* wound model can be used as an effective pre-clinical assay platform to investigate the effects of topical treatments on the wound healing process in chronic wounds. Importantly, different nutrient combinations affect tissue integrity, stability of the dermal-epidermal junctions, and even protein expression. For example, we have observed that DMEM culture medium supplemented with 10% fetal calf serum (FCS) and applied topically on *ex vivo* wounds increases the expression of VEGF and IL-10. Consequently, the use of more advanced culture media and replacement of nutrients derived from animal source can improve the investigation of human physiological responses. In this study, *ex vivo* human skin with standardized superficial wounds was cultured in four different media with different supplementation: i) DMEM with FCS, ii) DMEM with normal human serum (NHS) and an oxygen carrier (OC) of vegetal origin, iii) CnT-Prime™ medium with NHS and OC, and iv) EpiLife™ medium with NHS and OC. After a period of 12-days culture, we observed only a moderate time-dependent increase in lactate dehydrogenase (LDH) activity, while skin morphology was preserved and re-epithelialization occurred at the wound edges for all groups with the exception of the EpiLife+NHS+OC group. Starting from day 6, we noticed a loss of intercellular adhesion and disruption of the upper epidermal layers. The vascularization and re-epithelialization processes were monitored through the expression of CD31 and keratin-17, respectively. In general, moderate increased expression was observed for all groups over the incubation time but the EpiLife+NHS+OC group had the lowest values. This comparative study reveals important histological and functional effects of different culture media and suggests that culture media supplemented with NHS and OC can be suitable alternatives to media supplemented with serum derived from animal sources.

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Systematic establishment of a reproducible culture and defined differentiation procedures of colonic organoids

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The organoid culture is a great system to analyze biological processes and molecular mechanisms in an *in vivo*-like system. Organoids grow in three dimensions and form defined small cell clusters that mimic *in vivo* cellular functions to the greatest extent. This feature attracted an increasing number of researchers and expanded the range of applications, ultimately substituting and therefore reducing animal experiments. However, the culture is not well standardized and culture conditions vary between different laboratories. While small intestinal organoid culture relies on a relatively well-established method, the colon organoids were traditionally more difficult to grow and several protocols to maintain stemness in the colon have been developed. Colon organoids can now be cultured for long time in a particular maintenance medium, in which the proliferative stem cell-like cell state is highly enriched, while the culture lacks differentiated cells. This project aims to develop a standardized methodology for colon organoids that enables cellular differentiation in a tractable manner. Using a systematic approach, we defined a reproducible culture condition and defined differentiation procedures to enrich for the different epithelial cell populations of the colonic epithelium. These data will enable us to explore the signaling pathways that guide cellular differentiation and enable an adjustment of cell culture conditions for questions related to specific subtypes of differentiated cells.

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Quality enhancing measures in organ model research

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3D cell culture models are promising new approaches providing unique opportunities for the study of human diseases and treatment designs. However, proving the reliability and trustworthiness of this novel technology will be crucial for its broad acceptance and successful application in translational biomedicine. The *QUEST center for responsible research* is to evaluate current quality standards in 3D cell culture models. Here, we report on quality measures currently being established at the Einstein Center 3R (EC3R), a recently founded, Berlin based consortium of organ model experts.

We collaborate with researchers in the EC3R to increase the internal and external validity of their model systems and map out different dissemination strategies to support the 3R concept. By analyzing the transferability of organ models, we will evaluate challenges and define boundary conditions for the individual organ models.

To provide overviews on the current body of literature on human 3D cell culture systems, and assess the reporting quality in scientific publications towards replicability, we are conducting systematic reviews with members of the EC3R and additional partners. We will show preliminary data from a currently conducted large-scale systematic review analyzing the contribution of human organ models to COVID-19 research. Additionally, we are working on a systematic scoping review describing the state of the art in bioprinting of liver models in collaboration with bioprinting and liver experts.

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Brain organoids to model human brain diseases

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Understanding how the human brain functions in health and disease is one of the greatest challenges of modern science, yet hindered by limited availability of human samples and ethical restrictions. The three-dimensional human brain organoid model has emerged as a cutting-edge, genetically tractable experimental system to study human brain development and function in health and disease. Here, we will give an overview of recent developments in the MDC Organoid Platform and present one exemplary project illustrating how brain organoids can be employed to model human disease, specifically viral encephalitis.

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Der Simulierte Mensch (Si-M) – The Simulated Human

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In the research building "Der Simulierte Mensch" (Si-M), scientists from Technische Universität Berlin and the Charité Universitätsmedizin will work together to simulate the functions of human cells and tissues using new technologies of 3D cultivation, multi-organ chips or 3D bioprinting. In contrast to existing collaborative projects, the building will practice joint development of models "side by side" in the same laboratory environment and without the use of animal experiments.

The Si-M represents the goal of the two institutions Charité and Technische Universität Berlin to work more closely together and fits into the decided planning of a joint campus Seestraße. This campus will also be a signal for the formation of a cross-institutional 'scientific community' in the field of biomedicine and medical technology in connection with the joint initiative of the Berlin universities within the framework of the excellence strategy. The Si-M will act as a centre of communication between the two major research institutions.

"Der Simulierte Mensch" will serve as a catalyst in the joint development of human models and will serve as a continuous exchange between medical issues at the TU Berlin and technical requirements at the Charité. The construction is the essential prerequisite for the implementation of this specific program.

The research cooperation Si-M - at the intersection of engineering sciences and medicine - shall be scientifically leading in the development of innovative human model systems and new analytical processes of human cells and tissues. The key to success in the research program lies in the fusion at one location. The house stands as a symbol of a new way of thinking at the intersection of technology and medicine and will be perceived as such.

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3D Spacepatch: development of a 3D bioprinted wound patch for micro- und hypergravity conditions

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Wound healing represents a clinical challenge, not only on earth, but also especially under the extreme conditions during space flights. As conventional treatments, like autografting, are not feasible under these conditions, artificial wound patches could present a solution to enable endogenous healing. Specialized stereolithographic 3D Bioprinting systems allows for precise and fast fabrication of multi-component, personalized tissues in an automated manner, while offering a high reproducibility and printing quality in closed and automated systems and the possibility to be challenged under micro- and hyper-gravity conditions.

Here, we present a novel approach to evaluate such a wound patch system using a stereolithographic 3D bioprinted wound patch model (WPM).

The protocol of a published WPM was adapted and established for the process of stereolithographic 3D bioprinting by a careful selection of appropriate bioinks and crosslinking characteristics. Then, the wound healing extent of the 3D bioprinted WPM was assessed via immunofluorescent staining of skin maturation markers in comparison to a published WPM and to native skin. The extent of the skin maturation of both analyzed WPM show similarities concerning the intensity and spatial localization, suggesting a successful tissue maturation through cellular self-assembly processes. These results will be quantitatively validated with future qPCR analysis. Moreover, further analysis of WPMs bioprinted under different gravitational conditions during a parabolic flight will be conducted to validate a stable and robust bioprinting process under extreme gravitational conditions.

In summary, this proof-of-concept is not only the first step towards future wound treatments during space flights driven by cellular self-assembly, but also evidence for a very robust printing process even under extreme conditions. Taken together with the future exploration of potential optimizations, e.g. by introducing 3D interface architectures, it might as well lead to improvements in existing wound healing systems for the benefit of patients on earth.

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Complex human neuromuscular organoids as tools of disease modeling and personalized medicine approaches

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In the Gouti lab we have developed neuromuscular organoids from human pluripotent stem cells through an intermediate bipotent progenitor state, the neuromesodermal progenitors. Our neuromuscular organoids (NMOs) feature spinal cord neurons and skeletal muscle fibers that are generated in parallel, self-organize and interact functionally throughout the differentiation process. Our main focus is to enhance and accelerate the maturation process of NMOs in order to resemble the mature in vivo tissue. Here, we present our complex human NMOs with a particular emphasis on future technological developments. Towards this end, we have employed a multidisciplinary approach that exploits recent advancements in stem cell and organoid systems at the interface with new high-throughput and high-content imaging technologies, transcriptomics, optogenetics as well as bioelectronics. Our research also focuses on understanding neuromuscular diseases, such as Amyotrophic Lateral Sclerosis (ALS) and Smooth Muscle Atrophy (SMA), which are quite prevalent in the community and impose significant burden on patients and clinicians. Dissecting the complex mechanisms behind the onset and progression of such pathologies is a prerequisite for developing novel therapies and screening/discovering drug candidates to treat neuromuscular diseases. We anticipate that establishment of mature organoid models could contribute to replacement of animal models in the future and form the basis of a new platform for drug screening and personalized medicine approaches.

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Mechanisms of acquired chemotherapy resistance in pancreatic ductal adenocarcinoma in organoid models

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Pancreatic cancer represents one of the deadliest types of cancers with an overall survival rate of 10%. Effective treatment options are limited since pancreatic cancer shows high levels of chemotherapeutic resistance. Currently, up to 80% of patients suffer a relapse within the first five years.

In recent years, organoid technology has provided a promising platform for evaluating treatment response in PDAC. Up until today, however, organoid models have been used to evaluate drug responses at a single time point only. For a better understanding to be gained, this project aims at developing a dynamic predictive platform as well as understanding the dynamic changes in chemotherapeutic resistance.

In the first part of the project, 10 patient-derived organoid cultures from treatment-naïve patients will be established and characterized regarding histology, immunohistochemistry, DNA panel sequencing and treatment response. To induce resistance, the established cultures will be submitted to several cycles of chemotherapy closely resembling the clinical situation of the patients. We will identify mechanisms of resistance on a functional level by analyzing the transcriptome and proteome of the patients before, during and after the chemotherapy.

To address the question of whether these mechanisms are already present in a small subset of primary resistant cells before treatment or if they are induced in secondary resistant cells during treatment, we will establish single-cell-derived organoids. These organoids allow the identification of primary resistant clones within the heterogeneous treatment-naïve tumor.

In conclusion, our research project aims at developing a dynamic predictive platform for chemotherapy resistance in pancreatic cancer. This way, mechanisms of resistance can be identified and personalized drugs identified. In the long term, high-throughput drug screening in dynamic predictive platforms may allow future advances in pancreatic cancer treatment.

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Examining the differential tumor physiology in the primary tumor and metastatic sides in pancreatic cancer

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Pancreatic cancer represents one of the most common cancer entities in Germany and is predicted to be the second most common cause of cancer-related death by 2030. Due to its unspecific symptoms and aggressive tumor physiology, 50% of patients are already metastasized at the point of diagnosis. The 5-year survival rate in the metastatic stage is 2% only. Within the metastatic stage, however, patients with pulmonary metastasis live longer than those who present with a hepatic metastasis. Moreover, oligometastatic patients are known to live longer than multimetastatic patients.

In this research project, the differential tumor physiology of patient-derived organoid models of the primary tumor and the corresponding metastasis are being examined. Organoid models are verified histopathologically and genomically with the corresponding FFPE tissue of the patients. Once verified, immunohistochemical characteristics, the transcriptome and proteome of the organoid models of the primary tumor and the metastatic sides are examined. In a second step, the chemotherapeutic response of the individual models is analyzed via ATP-turnover (CellTiter-Glo®). The resistance to standard chemotherapeutic regimens (mFOLFIRINOX and gemcitabine/nab paclitaxel) is being determined. At the same time, the in-vitro response to treatment is compared to the radiological response of the primary tumor and metastatic side in the corresponding patient.

In summary, this project aims at establishing a predictive platform for a personalized therapy approach for metastatic pancreatic cancer patients. This way therapy decisions will not only be taking into account the metastatic stage, but more accurately the differential therapy response of the primary tumor and the different metastatic sides.

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Investigating the potential of patient-derived tumor organoids as a preclinical tool for predicting treatment response in patients with cholangiocarcinoma

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Cholangiocarcinoma (CCC) is an epithelial neoplasia of the hepatobiliary system and can be classified into intrahepatic and extrahepatic carcinoma. The 5-year survival rate is known to be very low (7 to 20%). Up until today, the only curative approach remains surgical resection. As the tumor is rapidly progressing, 20 to 30% of all patients present at a resectable stage with a probability of relapse reported to be up to 70% within the first 5 years. The majority of patients are diagnosed at an irresectable stage and are treated by palliative chemo-/immunotherapy. The availability of effective treatment options in the adjuvant as well as palliative therapy remain very limited. Having progressed in the first-line treatment (gemcitabine/cisplatin/durvalumab), molecularly targeted therapies have proven to be very successful (e.g. FGFR2 fusion/rearrangement, IDH1 mutation). 60% of all patients with cholangiocellular carcinoma, however, do not exhibit any targetable alterations.

In the context of this project, we are proposing the first ever prospective case series for the cholangiocellular carcinoma in which a total of 25 patient-derived organoids are isolated and established. In the prospective case series resection samples and biopsies of the primary tumor and the metastasis of both treatment-naïve and previously treated patients will be included. Histological, immunohistochemical and genomic analyses will be performed to ensure that organoids adequately represent the original patient samples. Subsequently, organoids will be exposed to chemotherapeutics and classified into sensitive, intermediate and resistant models. For the organoid model to be validated as an effective prediction platform, the radiological response rates after 12 weeks of therapy will be correlated to the in-vitro classification of the corresponding organoid models by using standardized RECIST-criteria. In Summary, this work aims to build up the first corner-stone for a personalized organoid-based therapy approach for patients suffering from cholangiocellular carcinoma.

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Development of a xeno-free human vessel-on-a-chip protocol

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Chip-based organoid models offer the opportunity of providing both, an alternative to animal experimentation and a more reliable bench-to bedside translation. However, these models often still rely on animal-derived products, such as serum or extracellular matrices, which apart from causing animal suffering may negatively influence the translation ability to human conditions. Here, we aimed to adapt a human vessel-on-a-chip model to a completely xeno-free protocol, covering all steps from cell culture to immunofluorescence staining.

We found that human umbilical vein endothelial cells (HUVEC) could readily be cultured in xeno-free medium. Intriguingly, HUVECs showed a more homogenous morphology under xeno-free conditions compared to the standard, animal-product containing culture conditions, highlighting the benefit of replacing animal products. Adapting the vessel-on-a-chip model to xeno-free conditions proved to be challenging. While initial vessel-like networks formed quickly, vessels were less stable after several days in the xeno-free protocol compared to the standard protocol. By reducing the concentration of growth factors and serum, the vessel stability could be improved.

High quality immunofluorescence staining was successfully conducted with xeno-free commercial blocking buffers and recombinant antibodies. As an increasing number of recombinant antibodies are becoming commercially available, it is feasible to replace all animal-derived antibodies in the future. However, the combination of antibodies that can be used simultaneously in one experiment is still limited due to the predominant rabbit-IgG form of the available recombinant antibodies and the lack of fluorophore-conjugated options.

In summary, we have established a xeno-free human vessel-on-a-chip protocol that can be adapted in future experiments to study specific diseases.

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Analyzing human lung organoid cultures of different origin for infectious disease research

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Background: 3D organoids generated from human lung tissue reflect the native cell composition of the lung as well as its physiological properties. The RKI aims to establish such lung organoid cultures as part of the establishment of an internal institute platform for the characterization of (novel) pathogens. In order to be able to guarantee a constant quality of the organoids, a quality control is required.

Objective: We aimed to compare lung organoids previously generated from human adult stem cells (two resources) and from commercially available lung cells in order to assess their suitability in terms of physiological relevance and short-term availability.

Methodology: To this end, we analysed the presence and abundance of cell types found in the lung by evaluating specific cell marker on RNA (RT-PCR) and protein level (Western blot). Furthermore, the correct spatial distribution of protein marker and cell types within the 3D organoid structure had been confirmed by visual observation applying CLSM.

Result: We found that the composition of cells in the organoid is comparatively consistent/equivalent with that in the primary lung tissue/material and in all tested organoids generated from varying sources. Furthermore, we challenged lung organoids with a mutant Ebola-Virus expressing GFP proofing for effective replication indirectly via the visualization of GFP and directly through the detection of the viral nucleoprotein on RNA and protein level.

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Vessels on-a-chip: A self-assembling vascular organoid model

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As 3R becomes more and more important, it is necessary to develop and establish functional in-vitro organ models. Reducing animal testing, they can be used for drug development and toxicology assessment, and hold great promise for regenerative medicine. However, a routine application of organoids is hindered by several limitations of the current models.

One critical limitation is the lack of a perfusable vascular system, which greatly limits their utility, especially for highly perfused organs such as the liver. We aim to solve this problem by introducing a self-assembling microvascular system, harnessing the intrinsic morphogenetic capacity of vascular endothelial cells. We develop a 3D-cell model, in which capillaries, generated by self-organized sprouting and tubulogenesis, form intact connections with organoids of e.g. liver tissue.

We investigate vessel maturation during vasculogenesis and angiogenesis and describe endothelial heterogeneity in the system over time. Further, we describe cell interaction and organ specificity of endothelial cells under the influence of surrounding cells as e.g., hepatocytes.

We trust that our approach will allow the generation of functional and mature perfusable capillaries for use in tissue/organ mimics.

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Modeling the tumor microenvironment in Head and Neck Squamous Cell Carcinoma organoids

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Patient-derived organoids (PDOs) are generated using 3D cell culture methods which maintain the self-organizing capacities of tumor cells and preserve the histopathological features of the parental tumors. Nonetheless, cell-type complexity is lost during serial passages *ex vivo*, resulting in PDOs cultures that lack the native tumor microenvironment. An air-liquid interface (ALI) culture technique for clinical tumor samples has been described (Neal *et al.* Cell, 2019), which allows to generate PDOs that maintain the cellular heterogeneity (tumor cells, stromal and immune cells) that constitutes the cancer ecosystem; making ALI-PDOs an optimal tool to mimic, at least in part, the complex interactions between these cells *in vivo*. In this study, we aim to adopt the ALI-PDO culture protocol in head neck cancers.

Five tumors were processed for both PDOs and ALI-PDOs. The ALI-PDO had an engraftment success of 40% (2/5 tumors), with an accentuated decrease in the number of ALI-PDOs upon splitting and time in culture. On the other hand, the PDOs protocol, had an engraftment success of 4 tumors (80%) with PDOs formation in 3 tumors (60%). Immunofluorescence analysis of the ALI-PDOs validated these were heterogeneous on their cellular content, with positive expression for EpCAM, Vimentin and CD45.

The culture protocol for ALI-PDOs was then altered to increase engraftment success and organoids expansion. Three tumors were processed for ALI-PDOs, where 2 tumors (67%) had ALI-PDOs formation and expansion, with 1 tumor (33%) being excluded by sample contamination. Immunofluorescence validated that the alterations were successful in increasing engraftment rates and organoid expansion, without major disruptions of cell-type heterogeneity.

In conclusion, we have taken the first steps to establish the protocol for ALI-PDOs culture in head and neck cancers. This will allow in the future testing the role of the tumor cell heterogeneity and tumor microenvironment in treatment response and resistance.

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Development of a bioreactor for the parallelized cultivation and stimulation of complex tissue models

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Musculoskeletal diseases are among the most common health problems. Particularly in the preclinical stage, animal models are still being resorted to, as no sufficiently suitable *in vitro* models are available. To close this gap, we developed a preclinical 3D *in vitro* model simulating the initial phase of fracture healing by co-cultivating fracture hematoma models (FH) with bone models. Although under non-perfused and unloaded conditions, our model demonstrates distinct overlaps between *in vitro* and *ex/in vivo*.

Since mechanical loading significantly influences the musculoskeletal system, we aim to develop a multimodal bioreactor capable of applying biomechanical loading that will allow us to cultivate 3D models *in vitro* under controlled and constantly monitored conditions.

Therefore, we defined mechanical loading conditions and applied these conditions to resemble the *in vivo* situation. Using this system, we mimic loading on models of bone regeneration. Finally, we test biomaterials and medication strategies under defined loading and environmental parameters.

Technically, we developed an insert-based cultivation chamber to incubate four models in parallel. We included a click-on pressure system to implement mechanical loading using a pneumatic valve. Using this system, we could prolong the surviving capacity of the cells in the *in vitro* 3D fracture hematoma models (FH) and accelerate the calcification of bone models consisting of self-assembled mesenchymal stromal cells.

The multimodal bioreactor proposed here will allow us to cultivate the model for a longer time under perfused and hence closer-to-*in-vivo* conditions and mimic loading conditions that influence the fracture healing process.

Approaching xeno-free cultivation of pulmonary pathogens *in vitro*

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In biomedical research, the cultivation of the bacterial pathogen *Streptococcus pneumoniae* requires products based on animal derived substances. Here we aim to reduce or replace these products with the intention of improving animal welfare and reproducibility. To establish alternative cultivation protocols, we screened xeno-reduced agar plates (Standard Nutrient Agar I) and xeno-free agar plates (CASO-Bouillon vegetal, Lysogeny Broth Agar vegetal, Standard Nutrient Agar I vegetal), as well as liquid growth media alternatives (containing CASO-Bouillon media vegetal, Lysogeny Broth media vegetal, Standard Nutrient Media vegetal) and human platelet lysate for optimal growth conditions comparable to existing methodology. To evaluate the applicability and integration of xeno-free and xeno-reduced products into bacterial cultivation methods, we selected the most promising candidates after screening combined solid and liquid phase growth conditions, followed by analyzing their capacity to induce cell death and stimulate cytokine production by epithelial cells.

The cultivation of *Streptococcus pneumoniae* serotypes 2 and 3 was successfully achieved through xeno-reduced and xeno-free alternative growth conditions, despite requiring longer incubation times and number of agar plates. *Streptococcus pneumoniae* serotype 3 demonstrated to be more accommodating to xeno-free growth than serotype 2 in the tested conditions. Flow cytometry analyses revealed that stimulation of A549 cells with different multiplicities of infection of *Streptococcus pneumoniae* serotypes 2 and 3, cultivated under xeno-free conditions, resulted in similar capacity to induce cell death compared to that of traditionally cultivated bacteria. Furthermore, the amount of interleukin-8 released into the cell culture supernatant by A549 cells during the 24-hour pneumococcal stimulation period revealed similar concentrations between traditionally cultivated and xeno-free cultivated bacteria, implicating no defect or enhancement of host-cell stimulation by use of alternative bacterial cultivation methods.

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Generation of a human 3D *in vitro* bone model that mimics glucocorticoid-induced osteoporosis

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Osteoporosis is a bone disease characterized by low bone mass and changes in bone architecture, often leading to pain, fractures and reduced mobility in affected patients. Glucocorticoid-induced osteoporosis (GIOP) is the best known form of secondary osteoporosis. To set up a GIOP *in vitro* model, we establish and characterize a human *in vitro* bone model, subsequently using methylprednisolone to induce GIOP and later treat the model with anti-osteoporotic drugs.

Our model includes osteoblasts and osteoclasts, which are mainly responsible for bone remodeling. We defined an osteoclast differentiation protocol using low-attachment plates and cultured the cells for 21 days in α MEM medium, 5% human AB serum, 2 mmol L-glutamine, 25 ng/ml M-CSF, and 50 ng/ml RANKL. To provide the basic scaffold for the structure of the "healthy" bone model, mesenchymal stromal cells were seeded on β -tricalcium phosphate (β -TCP).

Multinuclearity, typical β -actin ring formation, cellular activity by TRAP staining and functionality in resorption assays proved the functionality of osteoclasts. To establish the initially "healthy" (i.e., untreated) bone model, we seeded osteoclasts onto a pre-seeded β -TCP construct. We detected marked secretion of RANKL, MMP-9, and free phosphate. This indicates the functionality of both osteoclasts and osteoblasts in our 3D model. Subsequently, we will transfer our healthy model to the osteoporosis-simulating model where treatment will be applied. Once established, we plan to use the model for preclinical trials to test marketed drugs.

Ultimately, we will obtain an *in vitro* 3D co-culture of osteoblasts/osteoclasts simulating human native bone and mimicking key aspects of GIOP.

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***In vitro* investigation of the pathophysiology of *ex vivo* human brain tissue through acute and 3D culture models.**

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The use of animal models alone in the investigation of the brain represents a restriction to the complete understanding of the human brain physiology. In the attempt to overcome the architectural and functional differences between rodent and human brain, we established a research pipeline aimed to use state-of-the-art *in vitro* functional studies in *ex vivo* human brain tissue for translational research. This unique model is based on a multistep approach comprising: 1) acquisition of multiple sources of human brain tissue to allow a regular and robust supply; 2) centralized and optimized preparation of *ex vivo* human cortical and hippocampal tissue; and 3) distribution for both acute experiments and long-term 3D slice cultures. A near-sterile slice preparation protocol and an optimized transport condition allow us to provide samples of sufficient quality to perform different types of electrophysiological experiments in order to investigate both basic and translational research. Our work group has established the use of brain tissue in both acute and cultured conditions for several translational projects, e.g. characterization of autoimmune encephalitis-related human autoantibodies, organotypic slice cultures for viral and genetic manipulations, and disease models such as *in vitro* acute seizure-like events to study AMPA glutamate receptors involvement in epileptogenesis and antiseizure treatment. Despite the limitations due to the individual variability of each sample (age, biological gender, genetic background, pathological conditions) and the lack of a pure independent control, the use of *ex vivo* human brain tissue represents a strong translational model in the context of human brain pathophysiology.

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Characterization of the CDR1 as human iPSCs

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Circular RNA CDR1as (Cerebellar degeneration associated protein 1 antisense transcript) is a critical regulator of gene expression, known for its role as a miR-7 sponge, and is implicated in various diseases, including neurological disorders and cancer. In this study we aimed to generate, characterize, and analyze CDR1as knockout (KO) human induced pluripotent stem cells (hiPSCs) in brain organoids, providing a human-specific and ethically viable model system for studying CDR1as function.

We employed CRISPR/Cas9-mediated gene editing to deplete CDR1as expression from iPSC lines, using various knockout strategies: a) eliminating the CDR1as cassette or b) disrupting the 3' splice donor. Next we will examine the molecular consequences of CDR1as depletion, specifically focusing miR-7 regulatory network. Furthermore, we will explore the impact of CDR1as KO on iPSCs differentiation, and assess potential divergence between human and mouse models in the context of CDR1as function. We will report on this work in progress.

Embryonic hematopoietic-biliary liver organoids from human induced pluripotent stem cells as a model for development and disease

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In mammalian development, hematopoietic cells repopulate the embryonic liver in close proximity to the developing bile ducts. A better understanding of the hematopoietic-biliary co-development may reveal important mechanisms for infant leukemia, hemoglobinopathies, and cholestatic liver diseases. Here, we present the generation of hematopoietic-biliary liver organoids (HBLOs) from human induced pluripotent stem cells (hiPSCs), as a model to study liver development and response to early injuries of the liver.

To co-develop endoderm and hemogenic mesoderm derivatives in the same culture, we established a protocol that yields FOXA2+/CXCR4+ endoderm (62% of total cells) in symbiosis with a smaller population of KDR+/CD235a+ hemogenic mesoderm (6%) on day 4. Notch agonist JAG1 was used to promote biliary differentiation and a hematopoietic cytokine cocktail to induce hematopoietic differentiation. After embedding in Matrigel, the culture gave rise to CD34+/CD45dim(-) (1.1 %) hematopoietic progenitors in day 13 HBLOs. We multiplexed immunofluorescence of HBLOs and detected by confocal microscopy hepatoblast-like bipotential CK19+/HepPar1+ progenitors cells. Interestingly, only after adding hematopoietic cytokines, we observed biliary differentiation and morphogenesis into CK19+ luminal structures, even in the absence of biliary growth factors (i.e. EGF and HGF). These emerging embryonic portal-like structures also harbored alpha-SMA+ stellate cells, CD45+ leukocytes, and scattered IBA1+ macrophages reminiscent of the spatial composition of the embryonic liver. The addition of Biliatresone, a toxin that induces injury to developing bile ducts, resulted in elevated SOX17 expression accompanied by an increased number of biliary ductular-like structures, resembling the ductular reaction observed during cholestatic liver diseases.

In summary, our hiPSC-derived organoid system recapitulates key elements of the embryonic liver environment, enabling hematopoietic-hepatobiliary co-development in the dish. Our aims are now to improve further this system for elaborating a clinically relevant model of neonatal autoinflammatory cholangiopathies, or infant leukemia.

Human bone-like organoids to model the sequential stages of intramembranous ossification

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Conventional monolayer cultures can hardly reproduce all native tissue characteristics and functions. Here, organoids as advanced model systems offer a far more promising representation of human physiology and additionally serve as an alternative to the commonly used animal experiments, whose translational value may be challenging due to species-specific differences [1]. The combination of organoids and sensor technology through “Organ-on-a-Chip” devices enables the control and monitoring of physicochemical processes [2]. In this context, we intended to model the sequential maturation stages of intramembranous ossification and osteoblast-to-osteocyte differentiation by incorporating physiological parameters relevant to bone formation, such as mechanical stimulation and local oxygen tension [3]. Using carrier-free and carrier-based approaches, we established various human bone-like organoids from primary osteoblasts. First, we aimed to mimic the onset of intramembranous ossification by exploiting the ability of cells to self-assemble into larger agglomerates and pellets able to form ossification centers. Next, we used cell-laden type I collagen scaffolds to emulate bone matrix mineralization. Finally, a bio-printed cell-hydrogel allowed us to investigate the osteoblast-to-osteocyte maturation. By culturing these bone-like organoids for seven days in a self-designed “Organ-on-a-Chip” device, we were able to study the cellular response to a permanent perfusion, mechanical stimulation and adjustment of oxygen tension to a physiological range for bone (7% to 12%) [4]. We found that cells remain viable under all culture conditions and general metabolic parameters are affected differently by oxygen and mechanical loading. We also examined osteogenic parameters such as alkaline phosphatase activity and the release of prostaglandinE2 and nitric oxide. Furthermore, we assessed bone matrix calcification and the formation of osteocyte-typical prolonged cellular processes. We anticipate that future applications of bone-like organoids and their combination with “organ-on-a-chip” devices have the potential to replace animal experiments on bone biology in basic and applied research.

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Modeling neuroinflammation in brain-immune-assembloids to understand metabolic immune cell regulation after stroke

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Ischemic stroke is the most common form of stroke and is caused by occlusion of a cerebral artery. Consequently, undersupply of oxygen and nutrients lead to acute neurodegeneration at the lesion site. This acute cell death is followed by blood brain barrier disruption and peripheral immune cell infiltration. Accumulating immune cells within the brain display diverse neuroprotective and neurodestructive phenotypes, which can modulate neurodegeneration and outcome after stroke. The mechanisms that regulate this inflammatory cascade are still not fully understood. However, immune cell activation is associated with changes in glucose metabolism within the cell. In my PhD project, I will model neuroinflammation after stroke by combining human induced pluripotent stem cell-derived brain organoids and immune cells in in vitro stroke conditions. This brain-immune-assembloid model will be used to investigate metabolic mechanisms that regulate immune cell behavior and the inflammatory impact on neurodegeneration. By this, we aim to build an assembloid platform that can be used to identify key neuroinflammatory mechanisms in stroke to introduce new therapeutic opportunities for stroke treatment without relying on animal models.

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Cancer organoids for precision oncology and drug repurposing*Christine Sers¹, Ulrich Keilholz², Reinhold Schäfer²*

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Precision oncology seeks to implement therapeutic approaches predicted from patient omics profiles. This approach is often limited by emerging therapy resistance. Patient-derived model systems that represent patient profiles have the potential to test therapies experimentally and to investigate mechanisms of resistance preclinically. Previously, we have established extensive repositories of patient-derived organoids representing colorectal tumors (CRC) and melanomas (MEL). Extensive molecular characterization of CRC organoids^{1, 2} was performed within the international Oncotrack consortium. The generation and characterization of the MEL organoids was supported by the BMBF within the TREAT20 Plus consortium³.

Ongoing studies involving our organoid repository: 1) Finding novel approaches of multiple blockade of oncogenic signal transduction processes (so-called "vertical inhibition")⁴. 2) Preclinical companion study in parallel to off-label combination therapy of KRAS-mutated colorectal tumors based on co-inhibition of the RAS/ERK pathway and autophagy⁵. 3) Validation of cell-based drug screens to identify inhibitors against a signal-activated transcription factor⁶. 4) Molecular and functional characterization of cancer stem cells and oncogenic pathways at single-cell level^{7, 8}. To establish new organoids in a timely manner, we have described the logistical and experimental conditions in detail^{2, 9}. During bridging therapy, these 3D cultures may be subject to parallel testing of chemotherapeutics and targeted compounds that block tumor cell growth most efficiently. Existing organoid cultures can be used for benchmarking alternative organoid generation and testing, e.g. based on bioprinting and high-throughput analyses.

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Characterization of ex vivo cultured patient-derived glioblastoma explants for modelling the immune microenvironment and discovering potential resistance mechanisms to immunotherapies

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Introduction: Despite the success of checkpoint inhibition in various tumors, clinical studies have shown no significant survival benefit in glioblastoma multiforme (GBM) to date. One potential reason for this failure is the *in vitro* and *in vivo* models used in drug testing, which fail to recapitulate the complex biology of GBM. Patient-derived explants (PDEs) are small tumor fragments derived from original tumors that, in contrast to other models, retain three-dimensional, *in situ* information of the tumor immune microenvironment (TIME), which is essential for studying the different TIME elements and potential mechanisms of resistance to immunotherapies.

Methods: PDEs were used to characterize the tumor and immune cell compartment before onset of PDE culture and after 4 days in culture. To ensure good tissue quality, PDEs were assessed using hematoxylin & eosin (H&E) staining and later analyzed using immunofluorescence and immunohistochemistry. We performed imaging mass cytometry (IMC) of original tumors and corresponding PDEs of selected patients. Lastly, PDEs were treated with immunomodulating drugs used in GBM including dexamethasone, temozolomide (TMZ) and pembrolizumab to assess their impact on the TIME in our model.

Results: Tissue quality remained stable over the defined culture period and resident microglia and CD8+ lymphocytes could be identified in the cultured PDEs. IMC highlighted the variety of microglial phenotypes that are retained. Morphological assays define cell types of interest that can be further studied in PDEs.

Discussion: PDEs can model the TIME of individual GBM tumors and are amenable to reflect experimental manipulation of the TIME. Next, we aim to quantify drug distribution within PDEs. Ultimately, we plan to establish a pipeline for early therapy response evaluation and detection of resistance mechanisms utilizing the explant cultures as an “avatar platform”; upon disease progression and failure of first- and second-line treatment, PDE experiments could be used to inform decisions about alternative treatment options.

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Toward a human stem cell derived neuronal network for highthroughput cognitive drug screening: Characterizing the Functionality of Brain Organoids

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hiPSC-derived neuronal cell culture and brain organoids have been an emerging field since over a decade. The use of specific intrinsic and/or extrinsic factors has enabled researchers to develop a multitude of neural and glial cell types *in vitro*, and more recently brain organoid 3D humanized models that recapitulate healthy and pathological mechanisms of developing brains.

However, brain organoids have not been fully standardized nor characterized as a model for investigating synaptic mechanisms.

We investigate memory and forgetting mechanisms at the molecular, cellular and synaptic levels. Much of our work is conducted using mouse hippocampal slices due to it being a standardized model for studying such mechanisms. However, it is crucial that we also use a model that resembles human physiology as much as possible to increase the translatability of our research, whilst following and promoting the 3R principles.

So far, it has been shown that brain organoids express several neural and glial cell markers and show network activity in multi-electrode recordings. In the pursuit of identifying methods that promote synaptic strengthening (LTP) and weakening (LTD), we aim to first characterize brain organoids using immunohistochemistry to assess the presence of different neural and glial cell markers at different timepoints. Secondly, we are examining the immediate early gene marker cFos – a marker of cellular activity – to assess network activity of brain organoids subjected to different stimulated conditions including chemical LTP induced by briefly applying glutamate to promote long-lasting synaptic strengthening.

By characterizing when and to what extent brain organoids develop mature and functional network, we aim to establish a standardized hiPSC-derived brain organoid culture system that demonstrates synapse strengthening and weakening under different stimulatory conditions and can be used for high-throughput screening of cognitive drugs and validation of non-invasive brain stimulation methods.

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Modeling Colorectal Liver Metastases In Vitro: Utilizing Decellularized Human Liver Scaffolds as a 3D Platform for Cancer Research

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Colorectal cancer (CRC) is one of the most common cancer types worldwide, and up to 50% of CRC patients develop colorectal liver metastases (CRLM). Although liver resection is the only curative option, only a small fraction of patients are eligible for surgery. Despite advances in therapeutic strategies, the prognosis for CRLM patients remains poor. Therefore, it is necessary to develop and evaluate new in vitro models that accurately reflect tumor biology and metastatic state.

This study presents a patient-derived 3D in vitro model based on decellularized human liver scaffolds that closely mimic the native extracellular matrix. Non-tumor liver tissue was obtained from patients undergoing liver resection for CRLM, and the tissue was processed and decellularized. Histological and immunohistochemical staining for key matrix proteins confirmed the success of decellularization and assessed the extracellular matrix composition. The results showed that all cells were removed and the microstructure of the extracellular matrix was preserved.

HCT-116 or HT-29 colon cancer cells were then seeded on the decellularized liver scaffolds and incubated under standard cell culture conditions. The scaffolds supported the proliferation of both HCT-116 and HT-29 cells for up to 21 days in culture.

Furthermore, gene expression analysis revealed a significant increase in mRNA expression levels of known CRLM markers such as TIMP-1, MMP-9, SLC1A2 and VEGFA in the decellularized liver matrix compared to 2D cell culture conditions.

In conclusion, this study demonstrates the potential of patient-derived 3D in vitro models based on decellularized liver scaffolds as a valuable tool to improve our understanding of the tumor microenvironment and advance the development of new treatment options for CRLM patients.

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Artificial neural networks predict via label-free imaging analysis cellular injury in iPSC-derived human liver disease models

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Drug screenings on human disease models can be time-consuming and are often limited to detecting specific biomarkers. Artificial neural networks (ANNs) in imaging analysis have become a powerful tool for identifying phenotypic nuances at scale. Thus, they may overcome cumbersome screening read-outs to assess cellular injuries in a more global manner. Here, we test if ANN-guided, label-free brightfield imaging analysis could predict cellular injuries in iPSC-derived systems of different non-alcoholic fatty liver disease (NAFLD) models.

We tested different NAFLD injuries by exposing iPSC-derived hepatocytes (iHeps) to free fatty acids with or without extrahepatic signaling molecules relevant to NAFLD (“augmented injury”). We further exacerbated injuries by co-culturing iHeps with human peripheral blood mononuclear cells in transwells, mimicking steatohepatitis. We experimentally show in all injury conditions, that iHeps displayed similar levels of steatosis and significantly increased endoplasmic reticulum stress. The augmented injury further compromised mitochondrial function assessed via mitochondrial membrane potential. The steatohepatitis conditions aggravated mitochondrial dysfunction further. Using unstained brightfield images, we trained ANNs to distinguish cells from different hepatocyte injuries and let them define “injury scores”. For pictures not included in the training, the ANNs could identify all injury conditions from untreated cells with extremely high accuracy ($p < 0.001$). Strikingly, the ANNs could also distinguish cells from the different injury conditions, scoring cells of the augmented NAFLD model with higher injury scores than cells that underwent basal free fatty acid injury ($p < 0.01$). Extrapolating ANNs to the steatohepatitis conditions not used to train the model revealed higher injury scores, consistent with our experimental data. The injury scores correlated highly with mitochondrial dysfunction ($R = -0.84$, $p = 0.0006$). Thus, ANNs could distinguish different cellular injuries in iHeps that may permit their incorporation in large-scale drug screenings to identify novel treatment compounds straightforwardly.

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Development of a microfluidic organ-on-a-chip model for safety assessment of antiviral T-cell products

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In patients with end-stage organ dysfunction, solid organ transplantation (SOT) is the definitive treatment option. After successful transplantation, SOT patients are dependent on lifelong immunosuppression that prevent rejection of the transplant. However, treatment with immunosuppressants steers susceptibility to virus infections with e.g. cytomegalovirus (CMV) and Epstein-Barr virus (EBV). Classical antiviral drugs often fail to protect during severe disease course and have toxic side effects. Adoptive antiviral T-cell therapy represents a novel promising therapeutic approach that provides viral control while still guarding the transplanted organ. In the past, we have successfully established a GMPcompliant protocol to produce CMV- and EBV-specific T-cell products (TCPs). To verify the safety and efficacy of those TCPs current animal based models are not suitable. This is why, we are going to pave the way to a human based test platform by developing the essential building blocks for a multi organ-on-a-chip model comprising primary lung organoid cells and antiviral TCPs derived from the same donor.

For this purpose, a 2D lung epithelial cell monolayer culture was established in our hands, which can be directly transferred into a microfluidic organ-on-a-chip model. Our system is based on a monolayer established from singularized 3D lung organoids and allows combinatorial testing both co-culture dimensions. We have identified a suitable co-culture medium and coating to prevent re-formation of 3D organoid structures. In follow-up experiments, we will investigate the expression of lung epithelial markers and the MHC expression to verify sufficient viral antigen presentation to our TCPs. The ultimate goal is to transfer this monolayer into the organ-on-a-chip system that can comprise additional organs and allows evaluation of our TCPs in human based system. This platform could potentially replace less suitable animal models with a fully human autologous testing platform for a broad range of viral TCPs.

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Investigating the non-apoptotic role of caspases in endothelial mechano-adaptation using *in vitro* mechanical stress systems

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Caspases are proteases thought to be regulators and effectors of programmed cell death and inflammation, but new research reveals they may have additional unidentified functions. The goal of this study was to determine the potential functions of caspases in morphological adaptability of endothelial cells when subjected to known morphology-modulating stresses *in vitro*.

First, we subjected human pulmonary artery endothelial cells (PAEC, n=3 pooled) to a constant, unidirectional shear stress of 15 dyn/cm² in an *in vitro* fluid flow system for 72h. All known caspases were examined for modulation of endothelial morphological elongation parallel to flow through the addition of selective caspase inhibitors. We found morphological adaptation was functional in all situations, except with caspase-6 inhibition (74.8% adapted vs. 20.5% adapted, p=0.02).

To determine if caspase-6 necessity for shear-induced elongation was shear-specific or generally mechano-specific, we utilized an *in vitro* uniaxial cyclic strain system with a cyclic stretch of 5% at 1 Hz for 24h under caspase-6 suppression (n=3) as PAECs are known to elongate perpendicular to a strain axis. No difference in adaptation was detected between caspase-6-inhibited and control PAECs, demonstrating caspase-6 shear specificity.

Next, under caspase-6 suppression, micropatterned slides with thin RGD motif lines were employed as a patterning stimulus to drive PAECs into an elongated form. Caspase-6 did not impair morphological adaptation in this case, exhibiting shear specificity once more.

Finally, caspase-6 inhibition had no effect on cell adhesion and directed migration after the administration of an electrical or mechanical wound, indicating no role of caspase-6 in functional adhesive and migratory capabilities.

Our findings suggest that caspase-6 plays an important role in allowing PAEC to adapt to fluid shear stress specifically, which could have important implications for developing new targeted therapeutic approaches for cardiovascular diseases characterized by endothelial damage due to increased mechanical shear stress (e.g., reperfusion injury).

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3D bioprinting of perfusable organ models

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Three-dimensional (3D) organ models play a crucial role in medical research and drug discovery, providing insights into disease mechanisms and testing potential treatments. Traditional methods such as two-dimensional (2D) cell culture systems or animal models have limitations in replicating the complex structure and function of human organs in a humanized environment. Recently, 3D bioprinting has emerged as an advanced biofabrication technology with enormous potential in regenerative medicine and drug discovery, allowing the generation of 3D constructs using biological materials.

However, most current research on 3D bioprinted organ models focuses on static cultures, which hinder the recapitulation of dynamic microenvironments and substance delivery efficiency. Perfusion, which involves the delivery of nutrients and oxygen, is crucial for maintaining cell viability and function within 3D organ models, and is also vital for studying diseases such as cancer formation and pharmaceutical research such as drug delivery. Therefore, the development of perfusable in vitro organ models using 3D bioprinting has become an increasingly important research direction.

In this study, we aim to explore the potential of direct extrusion-based bioprinting for efficiently creating diverse perfusable in vitro organ models that closely mimic the in vivo microenvironment for biomedical research. The developed perfusable in vitro organ models have enormous potential in various areas of biomedical research, such as studying drug delivery, cancer progression, and the effectiveness of potential treatments. The ability to create perfusable in vitro organ models using direct extrusion-based bioprinting will enable researchers to study the interactions between cancer cells and the microenvironment, contributing to the field of bioprinting by exploring new possibilities and advancing cancer research by providing a novel tool for studying cancer.

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Kidney Tubule Organoids: Generating novel disease models from renal epithelial cells derived from patient urine

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There is a pressing need for more accurate models of the human kidney. The kidneys of animal models differ to those of humans in many respects including expression profiles, anatomy, and longevity. Human kidney cell-lines and primary-cells show markedly altered expression of key transport proteins and lack the diverse 3D-structure key to renal function. Although great strides have been made in kidney organoid development, the majority are complex to culture, poorly representative of the adult kidney, and lack the proliferative capacities necessary for applications such as high throughput screening.

In a 2019 publication, Clevers and co-workers described a protocol to generate simple 3D kidney tubule organoids – Tubuloids – from adult human nephrectomised tissue. These demonstrated expression profiles and transport activities akin to multiple sections of the nephron, though were predominantly proximal in nature. They also demonstrated tubuloids could be formed from renal epithelial cells (REC) derived from the urine of juvenile and adult donors.

Using the Clevers protocol as a foundation, we have developed a protocol to generate Tubuloids from RECs isolated from chronic kidney disease patients (CKD) that can be expanded and cultivated over multiple passages. Relative to the original, our method produces tubuloids more rapidly, with heightened cyst formation rates, and without contaminating cells (e.g. urothelia). We demonstrate these tubuloids express key markers of renal tubule identity, form polarized epithelia, and demonstrate transport activities expected of the proximal tubule. We also demonstrate our tubuloids are capable of recapitulating aspects of human kidney disease, as shown with tubuloids formed from the cells of a patient with polycystic kidney disease.

The ability to reliably generate easy to handle, patient-specific models of the human kidney from non-invasively acquired material would permit entirely new avenues of disease research and therapeutic testing, something we believe we are quickly approaching with our current work.