Major Research Project:

Animal-free matrix for organoids culturing

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Layman's summary

Organoids are a revolutionary tool in the field of biology that provides researchers with a unique way to study the behavior and function of different organs and tissues. Organoids are created by growing cells in a 3D environment that mimics the structure and microenvironment of a specific organ. This allows researchers to observe and experiment with the cells in a way that more closely resembles what happens in the human body. One of the key components of the organoid culture is the extracellular matrix (ECM), which is the environment that surrounds the cells and influences their physical characteristics such as shape, protein expression, polarization, and orientation of the cell. In many cases, the ECM used in the lab is derived from animal sources, such as Matrigel, which is used for liver organoids, and Basement Membrane Extract, which is used for kidney tubuloids. However, there are drawbacks for using animal-derived ECMs, including potential ethical concerns and the difficulty in obtaining a consistent, high-quality ECM. As a result, there is a growing interest in finding alternative, animal-free ECMs that can be used to culture organoids. This study aimed to evaluate the suitability of three animal-free alternatives, VitroGel, MaxGel, and BioGel for liver organoids, and VitroGel and MaxGel for kidney organoids, as potential replacements for animal-derived extracellular matrix. These materials have the potential to provide researchers with a more consistent and ethical way to study organoids and could lead to new discoveries and breakthroughs in our understanding of human biology.

Abstract

Organoids are three-dimensional cellular structures that mimic the architecture and microenvironment of a specific organ or tissue. The Extracellular matrix plays a crucial role in determining the morphology, polarization, and protein expression properties of the cells within the organ. Despite the widespread use of animal-derived ECM, such as Matrigel for liver organoids and Basement Membrane Extract for kidney tubuloids, there is a growing interest in animal-free ECM alternatives for organoid culture. This study aimed to evaluate the utility of VitroGel, MaxGel, and BioGel for liver organoids and VitroGel and MaxGel for kidney tubuloids as potential alternatives to animal-derived ECM. The cells were cultured over a period of 14 days with the alternative gels and the results were compared with the standard conditions, Matrigel or BME, based on the following characteristics: number, dimension, morphology, and function of the liver organoids and kidney tubuloids. The most important result was obtained using the MaxGel (Sigma-Aldrich) ratio of dilution 1:2 (MaxGel: DMEM F-12) which has the potential to be used for the maintenance of the tubuloids.

Introduction

Organoids mimic the characteristics of their original tissue and are able to maintain a stable culture and passage long-term. Organoids have emerged as a promising tool in disease modeling and drug discovery due to their ability to better mimic in vivo cellular organization, interactions, and functionality compared to conventional cell cultures (Fatehullah et al., 2016). Organoids are able to self-organize and differentiate into cell types that closely resemble those found in vivo, resulting in a more physiologically relevant model system. On the other hand, conventional 2D cell cultures can provide some valuable insights, but they fail to capture the complexities of tissue architecture and cell-cell interactions that are crucial to many physiological and pathological processes (Clevers et al., 2016). Organoids have a variety of potential applications, including modeling genetic diseases, drug discovery and toxicity assessment, cancer research, personalized medicine, and gene repair and transplantation therapy. Organoids can be used to develop new therapeutic strategies and may be a promising alternative to traditional organ transplantation. They have already been used to model a range of genetic diseases, including kidney disorders and neurodevelopmental disorders, and to screen drugs for cancer and infectious diseases. Organoids derived from individual patients can be used for personalized medicine, allowing for more targeted and effective treatments. In addition, gene editing technologies can be applied to repair genetic mutations in organoids, and organoids themselves may be used as donor tissues for transplantation (Tang et al., 2022).

Organoids can be generated from two types of cells, either adult stem cells (ASCs) or Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). (Lancaster et al., 2013). For organoids culturing are necessary growth factors and a 3D extracellular matrix gel that is similar to the microenvironment of the original. Organoids derived from adult stem cell-derived (ASCs) are composed of undifferentiated primary cells. ASCs have the capacity to differentiate into cells that are part of the organ or tissue they originate from. Compared to pluripotent stem cell-derived organoids, they are genetically more stable and have a lower probability of changes at the single-base level. (Huch et al., 2015). The higher genetic stability is due to the fact that PSCs- derived organoids have epigenetic abnormalities gained during the cell reprogramming and differentiation which is less frequent in ASC-derived organoids. (Ohnuki and Takahashi, 2015).

Disease modeling for liver and kidney

Disease modeling can use organoids for its purpose, in particular in this project we use liver organoids and kidney tubuloids. Liver disease is a major global health issue, responsible for approximately 2 million deaths annually (Mokdad et al., 2014). Chronic liver disease is characterized by constant inflammation and gradual deterioration of liver features, leading to poor quality of life, economic burden, and mortality risk (Stepanova et al., 2016). The prevalence of liver disease is increasing due to rising rates of behaviors and conditions such as alcoholism, obesity, diabetes, and viral infections. Alcoholism is a leading cause of chronic liver disease,

resulting in a 4.7% prevalence rate of alcohol-related liver disease in the United States, (Moon et al., 2020). Alcohol-induced liver disease describes a spectrum of symptoms that range from fatty liver to more severe conditions such as alcoholic hepatitis, cirrhosis, hepatic encephalopathy, and hepatocellular carcinomas (Lackner et al., 2017). Non-alcoholic fatty liver disease (NAFLD) is another syndrome that can lead to liver disease. In severe cases, NAFLD can progress to non-alcoholic steatohepatitis (NASH) and liver fibrosis (Sharma et al., 2022). Chronic viral hepatitis caused by HBV and HCV is the most common human cell-derived disease, affecting approximately 350 million individuals globally, with higher prevalence in Eastern Asia and Central and Southern Africa (Protzer et al., 2012). Non-treated chronic hepatitis C can lead to the development of hepatocellular tumors. Liver disease can also have genetic and autoimmune causes. The most common genetic causes are a deficiency in Alpha-1 antitrypsin and hemochromatosis, a disorder in iron absorption caused by an autosomal recessive disease. Wilson's disease, caused by copper accumulation, is another autosomal recessive disorder. Autoimmune hepatitis, PBC, and PSC are examples of autoimmune liver diseases (Sharma et al., 2022).

Chronic kidney disease is also a global health problem, affecting 10% of the population worldwide (Stein et al., 2021). This condition is characterized by the progressive and irreversible deterioration of the kidney's structure and function, leading to a reduced quality of life and increased risk of complications, particularly in the cardiovascular system, and higher mortality rates (Romagnani et al., 2017). To confirm the diagnosis of chronic kidney disease, the glomerular filtration rate (GFR) is assessed which is indicative of the quantity of blood that is filtered by the glomeruli of the kidney. A GFR measures how efficient is the kidney, a healthy adult kidney has a value of GFR that oscillates between 90 and 120 ml/min/ 1.73m^2 if the value is less than 60 ml/min/1.73m^2 there is evidence of kidney structure damage. Additional indicators of renal injury diagnosis include albuminuria, which is the presence of more than 30 mg of albumin in the urine in 24 hours or over 30 mg/g of albumin in a sample with urinary creatinine, as well as hematuria and leukocyturia, and other alterations in the minerals, imaging, and histology of the kidney (Romagnani et al., 2017).Chronic kidney disease has various causes, including diabetes, persistent hypertension, persistent use of anti-inflammatory drugs, inflammation of the glomeruli, autoimmune diseases, polycystic kidney disease, Alport disease, malformations, and the persistence of acute renal disease (Ammirati, 2020). Chronic kidney disease is classified into five stages based on the GFR and into three stages based on albuminuria (Ammirati, 2020). While chronic kidney disease is a significant global health problem, advancements in research and treatment strategies have improved the management of the disease, offering new hope for patients with this condition (Stein et al., 2021).

ECM and hydrogel

In the organ cells are located in a complex microenvironment that is characterized by multiple signaling interactions, mechanical forces, and the extracellular matrix (ECM). All these elements play a key role in the transformation, maintenance, and regulation of the cells (Prior et al., 2019). Hydrogels can simulate the natural ECM of the organ, influencing the biochemistry and physical properties of the cellular environment to drive some activities of the cells like cell adhesion, growth, migration, specialization, and interaction between cells-cell, and cell-matrix.

The porosity and hydration of the gel are important to supply growth factors and to ensure the normal function of the cells while maintaining the osmotic pressure. Additionally, the mechanical stiffness and viscosity of the gel matrix exert a significant impact on cellular behavior. The gel guarantees the normal passage of nutrients, metabolic wastes, and gasses among cells and between cells and the ECM (Liu et al., 2019). Hydrogels can be categorized based on their composition as natural, synthetic, and hybrid. Natural ECM can be animal or human-derived and is usually better suited for organoids since their components are more similar to the original ECM, On the other hand, synthetic hydrogels are made of materials that do not interact with the tissue and do not stimulate a response and can be modified to change their mechanical characteristics thanks to fixed compositions. Hybrid hydrogels, have both the characteristics of natural and synthetic hydrogels and is a promising approach to simulating the ECM. (Wieringa et al., 2018).

Matrigel is a commonly used hydrogel for organoid culture, made from the Engelbreth-Holm-Swarm mouse malign tumor cells enriched with extracellular matrix (ECM) proteins (Kleinman et al., 2005). It contains four primary components of the ECM, with the most prevalent being laminin at 60%, which can be present in different isoforms such as α1, β1, and γ1. Collagen IV is present at 30%, entactin at 8%, and heparin sulfate proteoglycan perlecan at 2%-3% These components provide adhesion sites suitable for various cell types, including stem cells, epithelial and endothelial cells, tumor cells, and induce differentiation and angiogenesis. Matrigel exhibits temperature-dependent properties, undergoing a phase transition from a liquid to a gel state between 22°C and 37°C. This transition is facilitated by the role of entactin, which serves as a crosslinker between collagen IV and laminin to enable gel formation (Aisenbrey et al., 2020).

However, Matrigel has several limitations. Its complexity and the more than 14,000 different peptides and the almost 2,000 proteins, led to property differences among different batches of Matrigel and within a single batch, making the reproducibility of experiments difficult. Furthermore, Matrigel is animal-derived, which presents several limitations, including difficulty translating to clinical use and ethical concerns related to the use of animals. The use of Matrigel in materials for clinical trials faces significant regulatory hurdles due to the inability to ensure the consistency and safety of the product. Therefore, it is challenging to obtain approval from the Food and Drug Administration (FDA) for the use of Matrigel in clinical trials (Jee et al., 201). The difference could also be found in the stiffness of the gel since the mechanical properties are the result of the elastic modulus (Aisenbrey et al., 2020).

Organoids on chip

Recent advances in microfluidics technology have enabled the design of highly customized systems capable of replicating *in vivo* environments for use in a vast use of different applications. By using a microfluidic system, shear stress, stretch, and other biomechanical forces can be simulated to create a more realistic environment (Saorin et al., 2022). Organ-on-a-chip is a model where the organs are represented by various cells that simulate the interaction between organs or tissues, it can be used for drug testing, and disease modeling and can be used as an alternative to animal models. They are made by seeding organoids in a 3D system where biological and physical forces are involved to create an *in vivo*-like environment. The cells

require oxygenation, and this is fulfilled by the constant circulation of the medium that also maintains the gradients of solutes (Saorin et al., 2022).

Aim of the project

The research question that guided this study was whether it is possible to find animal-free matrix alternatives for kidney and liver organoid culture that perform as well as Matrigel and BME. The hypothesis wanted to prove that from many animal-free matrix options that are available on the market for organoid culture, at least one of them should be suitable for organoids culturing. In order to test this hypothesis, the project involved testing different animal-free alternatives that are available in the market for liver organoids and kidney tubuloids. Three animal-free matrices were tested and compared to the laboratory standards, Matrigel for liver organoids and BME for kidney organoids. Despite the range of combinations tested, none of the hydrogels were found to perform as well as Matrigel and BME. However, it is fundamental to approach the comparisons critically and prioritize finding a hydrogel that optimizes the growth and function of liver and kidney tubuloids, rather than solely seeking similarity with established gold standards. It is important to emphasize the difference in the purpose of culture between liver organoids and kidney tubuloids. For the growth of liver organoids, a hydrogel that supports their growth and is also suitable for use in a microfluidic chip is necessary. In contrast, for kidney tubuloids, the matrix is only necessary for growth support.

Liver organoids

Liver organoids can be used for disease modeling, which is one of several approaches available. For example, people affected by Wilson’s disease have an abnormal accumulation of copper in various organs like the liver and the brain causing hepatic and neurologic disorders. Liver organoids have been used to study copper accumulation disease, in particular, this was caused by the mutation in the COMMD1 protein. Treatment with copper increased its accumulation compared to the wild type. Further studies have shown that an induced overexpression and transduction of a functional gene COMMD1 could reverse the phenotype. Another example of the use of organoids as a model disease could be their use for the study of ALD, which is caused by excessive alcohol consumption. Liver organoids can simulate the pathophysiology of the disease by co-culturing them with foetal liver mesenchymal cells and treating them with Ethanol. These organoids have shown increased activity of CYP2E1 and CYP3A4, a deposit of ECM, oxidative stress, and apoptosis (Nuciforo et al., 2020).

Matrigel is currently the standard for organoid culturing in our laboratory and is an extracellular matrix that is derived from the ECM of the Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Kleinman et al., 2005). However, Matrigel is not a consistent product and there are issues with supply, in addition to its being animal-based. Therefore, there is a growing need to find alternative, animal-free matrices for organoid culture. The use of Matrigel as a 3D scaffold in organoid culturing has greatly facilitated the development of many organoid models. However, its animal origin, batch-to-batch variability, and inconsistent composition are major limitations (Clevers, 2016). Moreover, the supply of Matrigel is not constant, and the cost can be prohibitive. To overcome these problems, researchers are actively seeking animal-free and

chemically defined alternatives to Matrigel (Chwalek et al., 2014). The ethical and practical benefits of using animal-free materials in organoid culturing are significant, as this can reduce the impact of animal use in research and increase the reproducibility and scalability of organoid production.

The cells used for the liver organoids like hepatocytes and ductal cells have a slower turnover rate, but they show a high regenerative capacity after liver damage (Aloia et al., 2019). Through the removal of a small tissue of the patient, we can obtain patient-derived organoids (PDOs) which could be done also with tumor tissue. Tumor organoids give an overview of the different characteristics of different subtypes of tumors. Organoids originating from a healthy liver tissue have the capacity to differentiate into a monolayer of epithelial cells, this cell can further differentiate into pseudo-lamellar epithelial cells, this potential of differentiation makes liver organoids a good instrument to investigate liver development, characteristics, and disease (Broutier et al., 2017).

Kidney tubuloids

The kidney is composed of complex functional units called nephrons, starting from the cortex and extending into the renal medulla. The nephrons are the functional units of the kidney responsible for the regulation of the level of electrolytes, acid-base, and homeostasis, the removal of waste products, drugs, toxins, and xenobiotics, and providing filtration while regaining electrolytes, nutrients, and liquids from the filtrate. Moreover, the kidney is also an endocrine organ and influences the sympathetic nervous system. There are more than 20 different cells in the kidney that can be divided into five main groups, with their own characteristics and functions (Yengej et al., 2020).

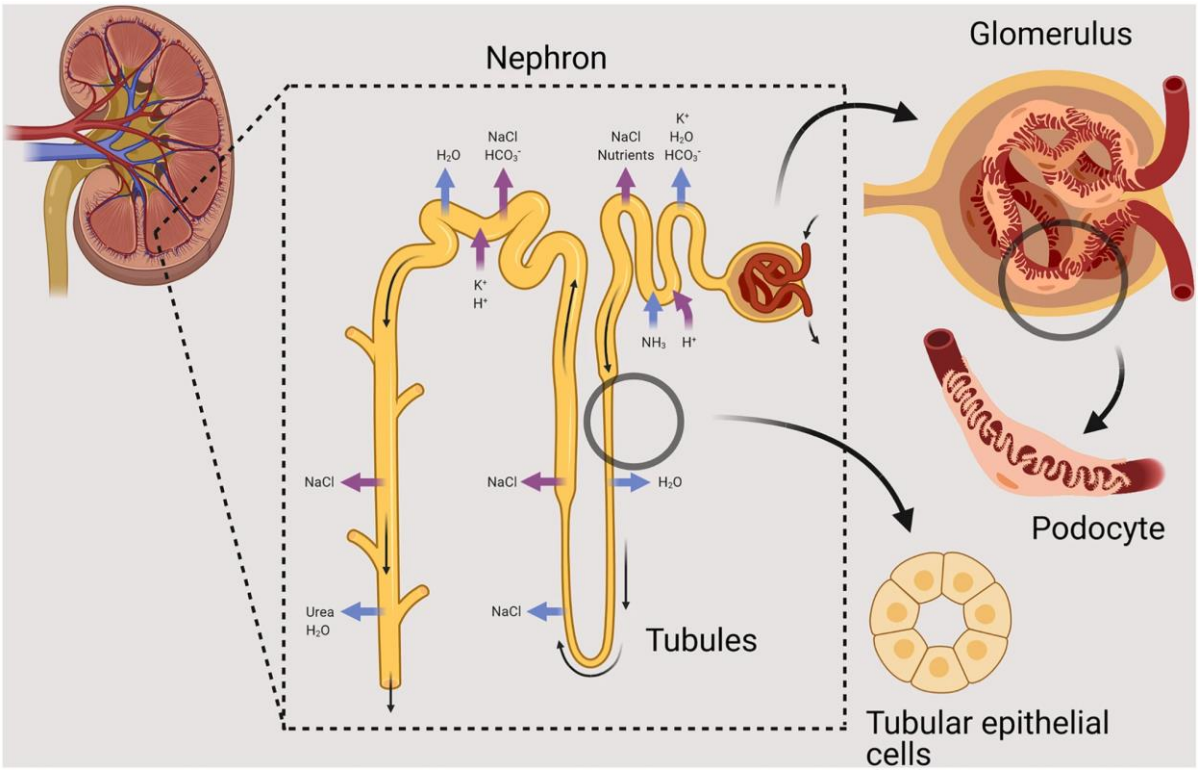

Figure 1. Overview of the physiology and anatomy of the kidney (Stein et al., 2021).

The complex architecture of the kidney, the multitude of different cells, and the elaborate function of the kidney need advanced tools to be replicated. Kidney organoids and kidney tubuloids can be derived from either pluripotent stem cells (PSCs) or adult stem cells (ASCs). Tubuloids are tridimensional multicellular cultures comprising adult kidney tubular epithelium. These structures are obtained by isolating epithelial cells from either kidney tissue or, less commonly, urine ((Schutgens et al., 2019; Yengej et al., 2020). To form kidney tubuloids, the primary renal epithelial cells need to grow in a basement gel that is the 3D environment, together with a growth medium. Epidermal growth factor (EGF) and fibroblast growth factor (FGF) stimulate the tyrosine kinase signaling while the R-spondin induces the Wnt amplification signal, the result is the stem cell state. Their gene expression, proliferation, and differentiation simulate renal plasticity *in vitro*. From single cells, RNA sequencing and immunochemistry is shown that there is the presence of structures such as polarized proximal and distal tubule, the loop of Henle, and the collecting duct epithelium. On the other hand, cells from the parietal epithelium and podocytes have not been detected. The presence of essential transporters has been shown by transcriptomics experiments and staining such as MDR3 and MDR4, OCT3, NKCC2, AE1, and AQP3 (Schutgens et al., 2019).

Experimental methodology and procedure

In this study, liver organoids were cultured with three different animal-free gels, and kidney tubuloids were cultured with two. At the end of the project, 44 different combinations were tested for liver organoids, and 30 different combinations were tested for kidney tubuloids. Liver organoids were cultured in a 96-well plate for 7 days in the hydrogel with liver growth medium, thereafter the medium was replaced with liver differentiation medium. On the 14^{th} day, different essays were performed on the liver organoids to verify the viability and functionality of the cells. Kidney tubuloids were cultured in the gel for 14 days with kidney growth medium, followed by the breakdown into single cells and then culture with kidney differentiation medium. Unlike liver organoids, kidney tubuloids do not require a hydrogel for integration into the microfluidic chip. However, the hydrogel is utilized during the culturing process to maintain cell viability. Liver organoids require an animal-free gel that allows growth and can be integrated into a microfluidic chip with constant flow, while kidney tubuloids utilize the hydrogel only for maintenance.

Material and Methods

Cell culture

The kidney tubuloids and the liver organoids were cultured in an incubator at 37°C degrees with 5% CO2 and 95 % humidity.

Liver organoids culture

Liver organoids were cultured in Matrigel in a 6-well-plate with liver growth medium, composed of Advanced DMEM F-12 complete (Advanced DMEM F12,1% v/v HEPES, 1% v/v Glutamax, 100 U/ml penicillin and100 µg/ml streptomycin) 2% v/v RSPO,1% v/v N2, 2% v/v B27,1% v/v Nic, 1,25 mM NAC, 100 ng/ml FGF-10 100, 10nM gastrin, 0.1% v/v primocin, 10 µM forskolin, 50 ng/m EGF, 25 ng/ml HGF, 5µM A 83-01. After 7 days of growth, the liver organoids were cultured with a differentiation medium that contained Advanced DMEM F-12 Complete, 1% v/v B27 1%, 1% v/v N2, 1,25 mM NAC, 100 ng/ml FGF-19,10nM Gastrin, 0.1% v/v Primocin, 25 ng/ml BMP7 25, 10 nM DAPT, 30 µM Dexamethasone, 50 ng/ml EGF, HGF 25 ng/ml, 5 µM A83-01 5 µ (Nguyen et al., 2022).

Liver organoids cultured with Matrigel were split every two weeks using cold DMEM F-12 applied directly to the hydrogel drops. In the beginning, a 1000 µl pipette is used to break down the organoids into small fragments thanks to the mechanical force and collected into a vial. The vial was centrifuged for 5 minutes at 1100 rcf at 4°C. The organoids were broken down again with a 200 µl pipette, washed with cold DMEM F-12, and centrifuged for another 5 minutes at 1250 rcf at 4°C. The cold Matrigel was then mixed with the cells and transferred into a 12-well plates.

Renal tubuloids culture

Kidney tubuloids were cultured in Basement Membrane Extract Culturex^{tm}(Bio-techne) in a 6-well plate with kidney growth medium, composed of Complete Advanced DMEM F-12 (Advanced DMEM F12, 1% v/v HEPES, 1% v/v Glutamax, 100 U/ml penicillin and 100 µg/ml streptomycin), 1mM RSPO,1.5% v/v B27, v/v,1% v/v R-Spondine 3, 50 ng/ml EGF, 100 ng v/v FGF-10, 10µM Rho kinase inhibitor, 5µM A-83-01(Nguyen et al., 2022).

Kidney tubuloids were split every 2 weeks for propagation. The gel was washed with cold Complete advanced DMEM F-12 and broken down into small fragments by the mechanical action of a 1000 µl pipette. The tubuloids were collected in a sample and resuspended with cold Complete advanced DMEM F-12 and centrifuged at 1200 rcf for 5 min at 4°C. The supernatant was discharged and resuspended with Complete Advance DMEM F-12 and tubuloids were broken down a second time but with a 200 µl pipette, and then centrifuged at 1500 rcf for 5 min at 4°C. Subsequently, the supernatant was discharged and the kidney tubuloids were cultured in a 6-well plate using cold BME.

Types of hydrogel	*Concentration of hydrogel*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*
VitroGel 1	100%	3:1	2:1	1:1	1:2	1:3
VitroGel 2	100%	3:1	2:1	1:1	1:2	1:3
VitroGel 3	100%	3:1	2:1	1:1	1:2	1:3
VitroGel 4	100%	3:1	2:1	1:1	1:2	1:3
MaxGel	100%	3:1	2:1	1:1	1:2	1:3
PeptiGel Alfa 1	X	X	2:1	1:1	1:2	1:3
PeptiGel Alfa 1 RGD	X	X	2:1	1:1	1:2	1:3

Table 1. Liver organoids cultured VitroGel, MaxGel, and Peptigel with different ratios of dilution (hydrogel: DMEM F-12).

Types of hydrogel	*Concentration of hydrogel*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*	*Hydrogel: DMEM F-12*
VitroGel 1	100%	3:1	2:1	1:1	1:2	1:3
VitroGel 2	100%	3:1	2:1	1:1	1:2	1:3
VitroGel 3	100%	3:1	2:1	1:1	1:2	1:3
VitroGel 4	100%	3:1	2:1	1:1	1:2	1:3
MaxGel	100%	3:1	2:1	1:1	1:2	1:3

Table 2. Kidney tubuloids were cultured with VitroGel and MaxGel with different ratios of dilution (Hydrogel: DMEM F-12).

VitroGel 3D culture

Liver organoids and kidney tubuloids were cultured in VitroGel 3D (The Well Bioscience inc.), an animal-free hydrogel that can simulate the environment of cells in the organ. The gel is polysaccharide-based, has neutral pH, and a room temperature jellification. Four different hydrogels have been evaluated, each having different components and mechanical properties for organoid culturing. The mechanical strengths of the different VitroGel hydrogels are in this order: VitroGel 3 > VitroGel 4 ≥ VitroGel 2 > VitroGel 1.

Liver organoids were cultured with room temperature VitroGel. The cells were incorporated into the hydrogel, rather than simply applying the hydrogel, or diluting it with varying ratios of Advanced Complete DMEM F-12. Successively, the cells were added to a 96 cell culture well plate. After 30 minutes of incubation, the growth medium was added.

MaxGel culture

Liver organoids and kidney tubuloids were cultured in human-derived MaxGel (Sigma-Aldrich) that emulates the ECM giving a three-dimensional environment to the cell. MaxGel is an animal-free matrix, generated from the co-culture of human fibroblast and epithelial cells *in vitro*, and can imitate the interactions between epithelial and mesenchymal cells. The hydrogel comes in a liquid form and a neutral pH, and the gelation occurs at room temperature.

The hydrogel was added into the vial with liver organoids or kidney tubuloids, directly or diluted with different ratios of Advanced Complete DMEM F-12, and subsequently added into a 96-well plate. The cells were incubated for 30 minutes, and after this period growth medium was applied.

PeptiGel culture

Liver organoids were cultured using the synthetic hydrogel PeptiGels (Manchester BIOGEL), which is capable of emulating the cellular environment and facilitating 2D and 3D cell growth. The PeptiGel product line comprises four distinct hydrogels, each with an enriched version containing fibronectin. The PeptiGel hydrogels possess several favorable attributes, such as biocompatibility, transparency, and ease of use. However, the constant jelly-like consistency of the PeptiGel necessitates careful addition to the cells to prevent bubble formation. In this study, two PeptiGel variants, PeptiGel Alfa 1 and PeptiGel Alfa 1 RGD were employed in combination with Advanced Complete ADMEM F-12 to generate eight unique hydrogel combinations with varying mechanical properties.

Functional essays

Rhodamine 123 essay

To verify the functionality of the membrane transporter Multidrug Resistance Protein 1 (MDR1), half of the liver organoids in a plate were incubated with 10 µM of the inhibitor Verapamil (Sigma), diluted with Advanced complete DMEM F-12, while the other half was incubated with HBSS for 30 minutes. After discarding the medium, organoids were incubated for 20 minutes with 100 µM Rhodamine (Sigma), diluted in liver growth medium. The medium was discarded from the plate and the organoids were washed six times with PBS. The organoids were then taken to the EVOS FL cell Imaging system (EVOS FL Auto, Life Technologies) where the green fluorescence signal was shown.

Quantitative PCR analysis

RNA Isolation

After culturing liver organoids with the hydrogel with Liver growth medium for one week and liver differentiation medium or liver growth medium for another week, the hydrogel was discarded

and Trizol was added for RNA isolation. In the next step, chloroform was added to the mixture in a 1:3 Trizol: chloroform ratio, followed by incubation for 5 minutes at room temperature and centrifugation at 12,000 rcf for 5 minutes at 4°C. The aqueous phase was then discarded, and 1 µL of Glycogen Blue was added to make the RNA visible. After adding 500 µL of isopropanol, the mixture was incubated at room temperature for 10 minutes and then centrifuged at 12,000 rcf at 4°C. The supernatant was discharged, and 760 µL of 75% Ethanol-DEPC was added. The mixture was centrifuged at 12,000 rcf for 5 minutes at 4°C, and the supernatant was again discarded. The pellet was then dried at room temperature for about 15-20 minutes, after which 10 µL of ultra-pure water was added. Finally, a 1 µL sample was taken to measure the quantity of RNA content on a Spectrophotometer DS-11 (DeNovix).

cDNA synthesis

After calculating the quantities for the cDNA synthesis, the SensiFAST cDNA synthesis kit (Meridian) was used to reverse transcript 500 ng of the isolated RNA. The reaction volume for each sample was 20 µL, consisting of 1 µL reverse transcriptase, 4 µL reverse transcriptase buffer, and the remaining volume consisting of RNA diluted in MQ water to a final volume of 15 µL. The reaction vials were then inserted into the T100 Thermal cycler (Bio-Rad).

Quantitative PCR

To assess the gene expression of liver organoids, a Real-time(qPCR) was conducted. The cDNA of liver organoids was mixed with primers, the Master Mix SYBER green (Bio-Rad), and water. Subsequently, the resulting samples were loaded into a 96 well-plate for PCR and subjected to qPCR analysis using the CFZ Real-time system (Bio-Rad). Specifically, the expression levels of the following genes GAPDH, CYP3A4. ALB, MRP2, CYP3A4, and CYP1A2 were quantified using the qPCR.

Primer	Forward	Reverse
GAPDH	5′-TGCACCACCAACTGCTTAGC-3′	5′-GGCATGGACTGTGGTCATGAG-3′
CYP3A4	5′-TTTTGTCCTACCATAAGGGCTTT-3′	5′-CACAGGCTGTTGACCATCAT-3′
ALB	5′-GTTCGTTACACCAAGAAAGTACC-3′	5′-GACCACGGATAGATAGTCTTCTG-3′
ABCC2 (MRP2)	5′-CCCTGCTGTTCGATATACCAATC-3′	5′-TCGAGAGAATCCAGAATAGGGAC
CYP3A4	5′-TTTTGTCCTACCATAAGGGCTTT-3′	5′-CACAGGCTGTTGACCATCAT-3′
CYP1A2	5′-CCCAGAATGCCCTCAACA-3′	5′-CCACTGACACCACCACCTGAT-3′

Table 3. Quantification of liver organoid genes was performed using quantitative polymerase chain reaction (qPCR).

Results

In this study, liver organoids were cultured with three different animal-free gels, and kidney tubuloids were cultured with two. At the end of the project, 44 different combinations were tested for liver organoids, and 30 different combinations were tested for kidney tubuloids. Liver organoids were cultured in a 96-well plate for 7 days in the hydrogel with liver growth medium, thereafter the medium was replaced with liver differentiation medium. On the 14th day, different essays were performed on the liver organoids to verify the viability and functionality of the cells. Kidney tubuloids were cultured in the gel for 14 days with kidney growth medium, followed by the breakdown into single cells and then culture with kidney differentiation medium. Unlike liver organoids, kidney tubuloids do not require a hydrogel for integration into the microfluidic chip. However, the hydrogel is utilized during the culturing process to maintain cell viability. Liver organoids require an animal-free gel that allows growth and can be integrated into a microfluidic chip with constant flow, while kidney tubuloids utilize the hydrogel only for maintenance.

Liver organoids

VitroGel

Liver organoids were cultured with VitroGel 3D, for a period of 4 days. A total of 6 different gel: medium ratios were tested for the 4 different types of VitroGel. The finding indicated that the combination that showed some outcomes were, for Organoid-1 were either pure VitroGel or a 3:1 ratio (VitroGel: DMEM F-12), while a 3:1 ratio (VitroGel: DMEM F-12) was found to give some results for VitroGel-2 and VitroGel-3. For VitroGel-4, the combinations that permitted some growth were either pure hydrogel or a 2:1 ratio (VitroGel: DMEM F-12), figure 2. The minimum results for those combinations were determined based on multiple parameters, including the number of organoids that developed within a 14-day timeframe, the rate at which they grew, the maximum size achieved, and their morphology. These criteria were used to evaluate and select the combinations that were most effective for supporting the growth and development of the organoids.

Based on the observation depicted in figure 2 and figure 3, the number of liver organoids cultured with VitroGel 3D appeared to be higher than those cultured with MaxGel and PeptiGel, but comparable to those cultured with Matrigel. The liver organoids had a complex morphology that was not spherical in shape, and some appeared stretched. Compared to organoids cultured with Matrigel, those cultured with VitroGel 3D tended to be smaller from the beginning and showed a slower growth rate after one week. The VitroGel 3D gel was observed to be softer and less stiff in comparison to Matrigel in all dilution combinations and it was not able to form droplets in the plate.

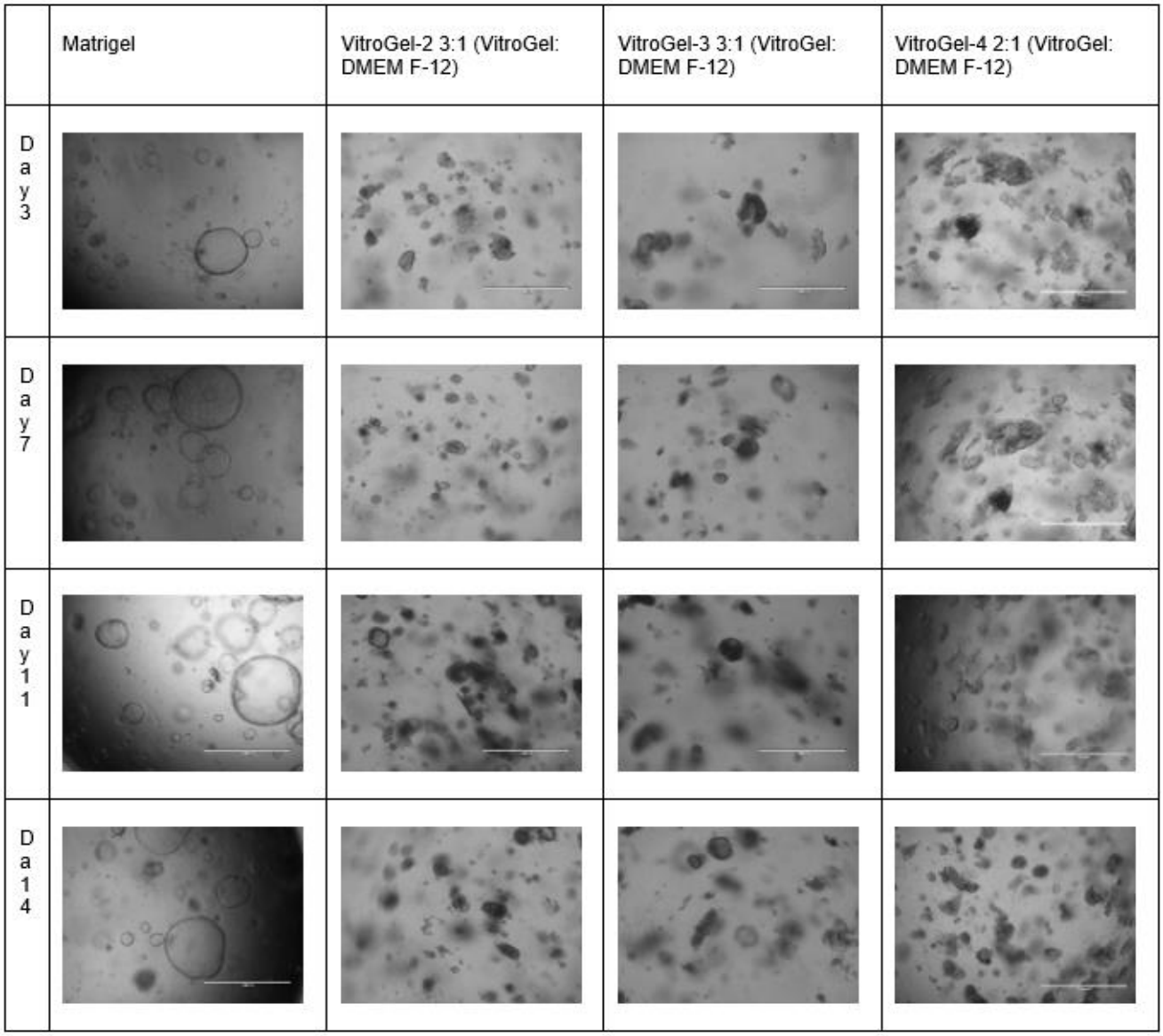

Figure 2. Liver organoids cultured with Matrigel, VitroGel Bottle 2 ratio 3:1 (VitroGel: DMEM F12), VitroGel Bottle 4 ratio 2:1, VitroGel bottle 3 ratios 3: at day 3, day 7, day 11, and day 14. The figure illustrates the temporal growth of the liver organoids and the noticeable variation in size and morphology across organoids cultured with the different dilutions of the VitroGel.

MaxGel & PeptiGel

Liver organoids were cultured with MaxGel (Sigma-Aldrich) for 14 days. For this hydrogel, 6 different combinations were tasted, with the ratio of dilution of 3:1, 2:1, and 1:2 (MaxGel: DMEM F-12) showing some results compared to the others, and the ratio of 1:2 (MaxGel: DMEM F-12) yielding the best results. Figure 2 and Figure 3 showed that the number of liver organoids cultured with MaxGel was lower compared to those cultured with Matrigel or VitroGel. The morphology of the organoids tended to be spherical and larger over time compared to those cultured with VitroGel, but smaller compared to those cultured with Matrigel. The hydrogel tent to form a monolayer, and the formation of domes was not observed.

Liver organoids were cultured with PeptiGel (Machester BioGel) for 14 days. For this hydrogel, 7 different combinations were evaluated with three being PeptiGel Alfa 1 ratio of dilution (2:1, 1:1, and 1:2) and PeptiGel Alfa 1 RGD, enriched with fibronectin, with ratios of dilution (1:1, 2:1, and 1:2). The PeptiGel Alfa 1 ratio of 1:2 (PeptiGel: DMEM F-12) and PeptiGel alfa 1 RGD ratio of 1:1 (PeptiGel: DMEM F-12) have shown some minimal results among the other combination. As is shown in the comparison between Fire 2 and Figure 3, the cells had a rounded shape and tended to grow larger compared to those cultured with VitroGel. The consistency of PeptiGel was distinct from the other hydrogels, appearing as a stiff and viscous gel. The gel formed zones with varying densities within the well, with cells tending to cluster in the areas with higher gel density. The stiff consistency of the gel allowed for the formation of domes.

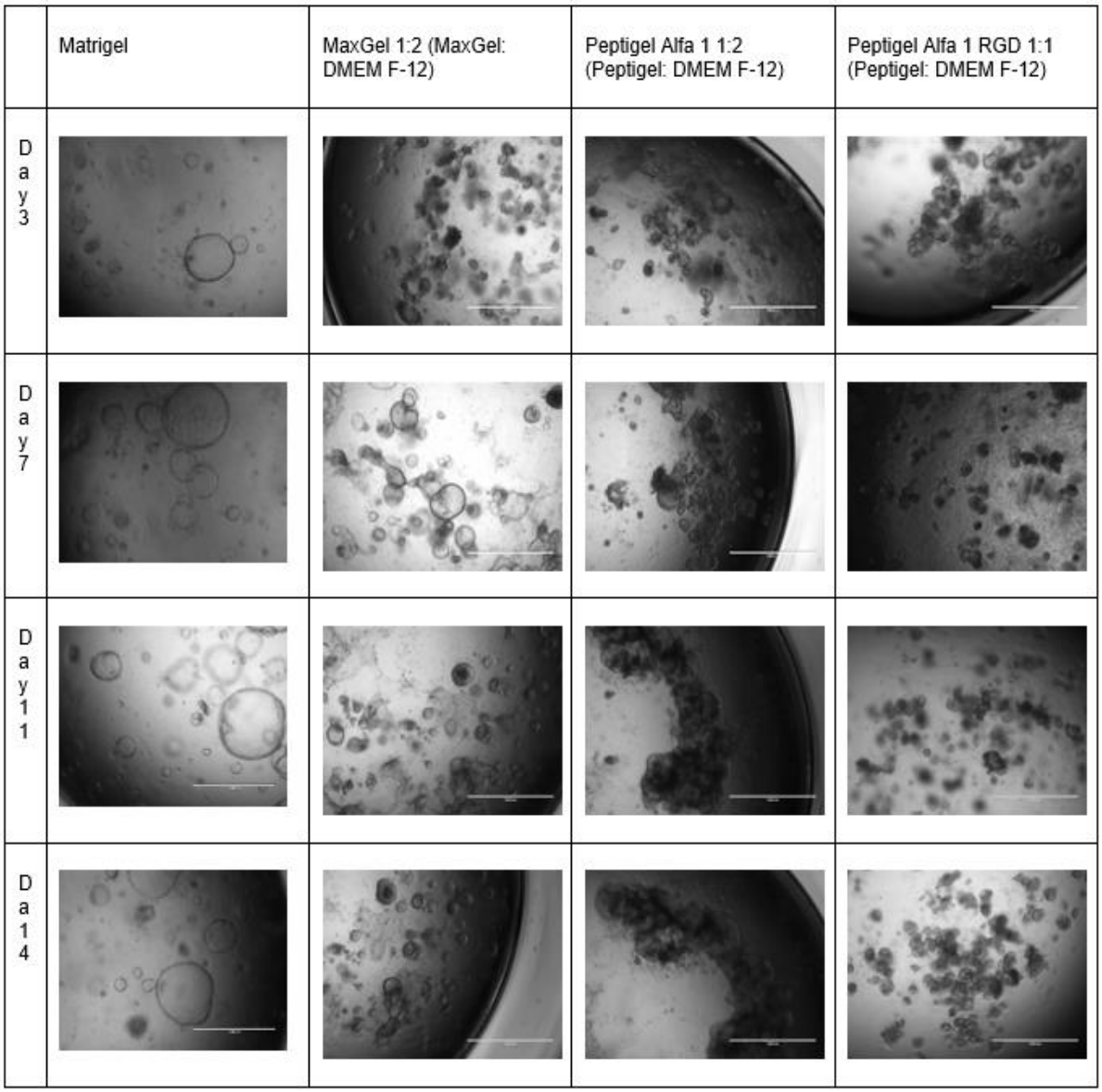

Figure 3. Liver organoids cultured with Matrigel, MaxGel ratio 1:2 (MaxGel: DMEM F-12), PeptiGel Alfa 1 ratio 1:2 (PeptiGel: DMEM F-12), PeptiGel Alfa 1 RGD ratio 1:1, at 3, day 7**,** day 11, and day 14. The figure shows the temporal growth of the liver organoids and the variation in size, morphology, and number across organoids cultured with the different dilutions of PeptiGel and MaxGel.

Rhodamine essay

Liver organoids were incubated with a fluorescent substrate of the receptor multi-drug resistance 1 (MDR1), the Rhodamine 123. Under normal conditions, the substrate accumulates inside the organoid lumen and the fluorescence can be observed using a fluorescence microscope. If the organoids are cultured with the competitive inhibitor Verapamil before incubation with Rhodamine, the fluorescence in the organoid lumen will not be present, since it will remain inside the cells. This Rhodamine assay demonstrates the functionality of the MDR1 receptor in liver organoids.

As seen from the results in Table 7, the organoids cultured with MaxGel, in the absence of Verapamil, exhibit fluorescence in the lumen, similar to those cultured with Matrigel. The organoids cultured with VitroGel, in the conditions of the absence of Verapamil, may display fluorescence similar to Matrigel or display multiple zones of fluorescence within one organoid, suggesting the presence of multiple lumens. On the other hand, the liver organoids that were cultured with PeptiGel showed minimal presence of Rhodamine even in the absence of Verapamil, with the signal being observed in only small portions of the organoid.

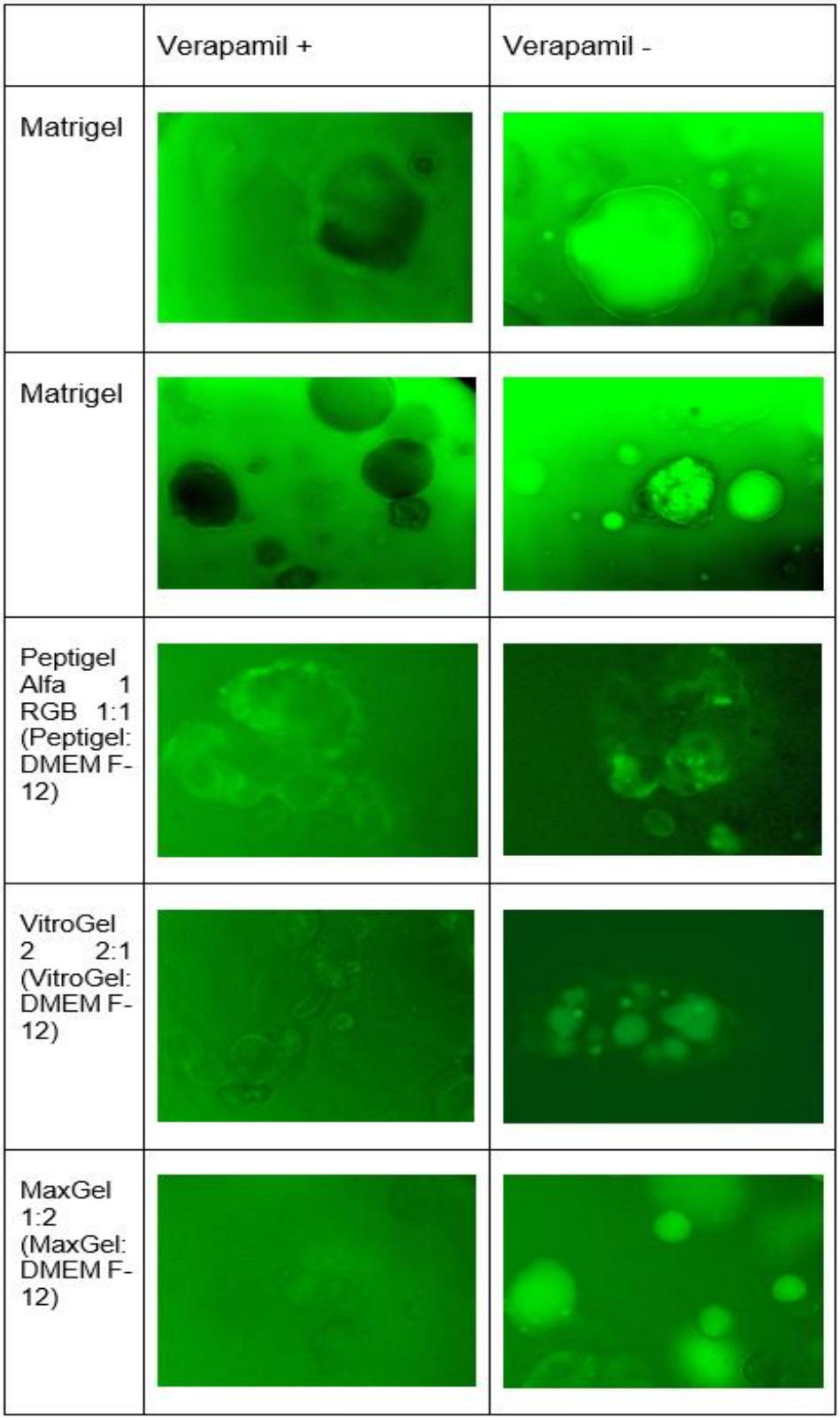

Figure 4. Liver organoids were cultured with matrigel and three animal-free gels different gels and submitted to the Rhodamine essay. The cells were cultured with Matrigel, Peptigel Alfa RGB ratio 1:1 (PeptiGel: DMEM F-12), VitroGel 2 ratio 2:1 (VitroGel: DMEM F-12), MaxGel ratio 1:2 (MaxGel: DMEM F-12). In the column on the left organoids were pre-incubated with the inhibitor Verapamil.

Kidney tubuloids

Kidney tubuloids were cultured with VitroGel and MaxGel for 14 days. Similar to the liver organoids, four different bottles of VitroGel were used, each with six different dilution combinations. As for the liver organoids, the optimal combinations of hydrogels for organoid culture were determined based on several key parameters that can be easily identified using the microscope, which include the quantification of organoid number and size over a 14-day time period, as well as their growth rate and overall morphology. These factors were evaluated to determine the combinations that were most effective in supporting the growth and development of the tubuloids. Compared to the tubuloids cultured with BME, those cultured with VitroGel showed fewer numbers and smaller sizes. Their shape was complex and not spherical, with slower growth observed after one week of culturing. The hydrogel was soft, with a consistency that did not allow for droplet formation as observed with BME. As shown in table 8, the best results were obtained with the 3:1 (VitroGel: DMEM F-12) and 2:1 (VitroGel: DMEM F-12) ratios of VitroGel-1 and VitroGel-2, respectively.

The kidney tubuloids were also cultured with six different dilution ratios of MaxGel, with the best results obtained with a 1:2 (MaxGel: DMEM F-12) ratio. The organoids had a round shape and were larger than those cultured with VitroGel, but smaller compared to those cultured with BME. The MaxGel tended to form a single layer, with zones of different densities observed over time, affecting cell arrangement.

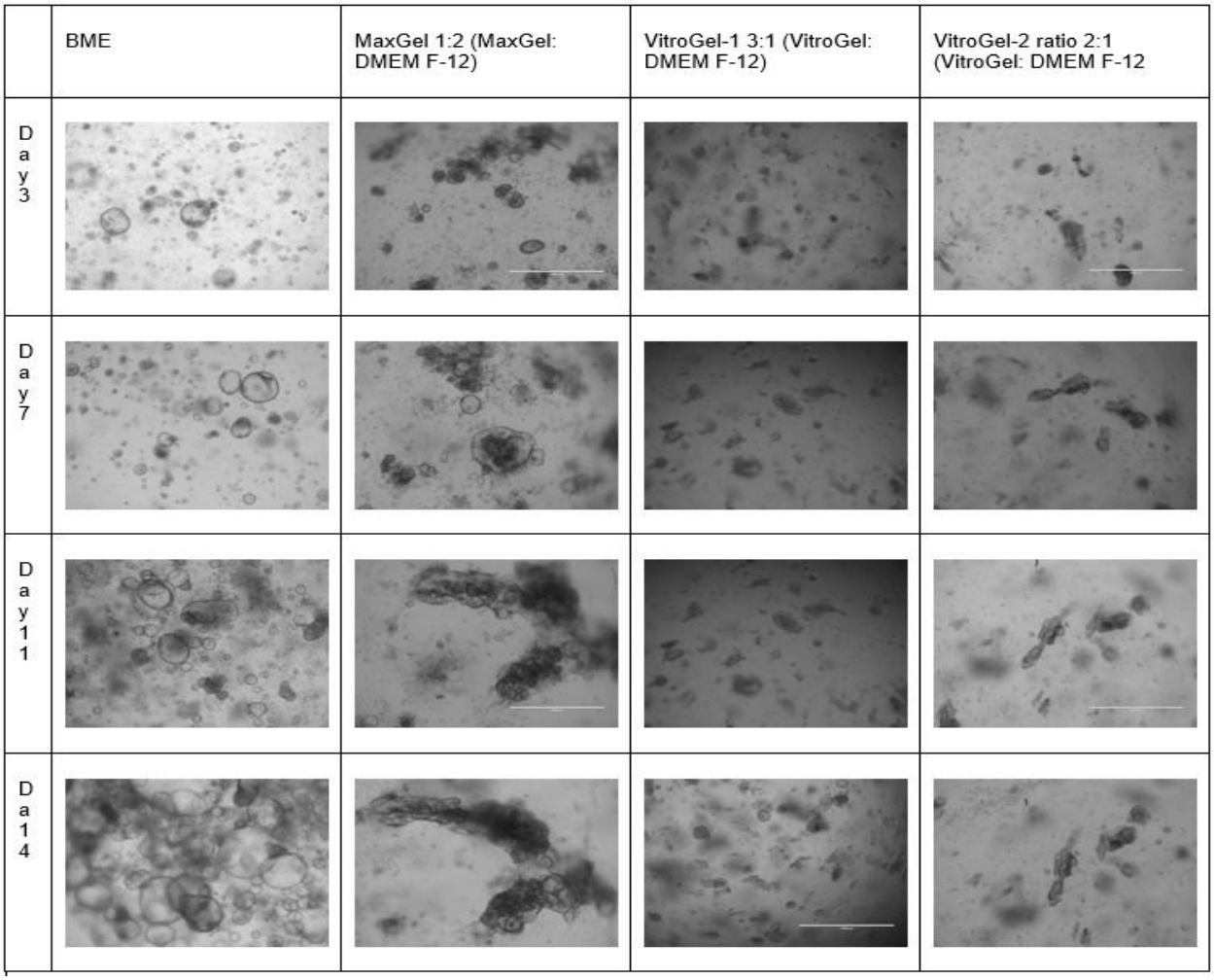

Figure 5. Kidney tubuloids were cultured with BME, MaxGel ratio 1:2, VitroGel-1 ratio 3:1 (VitroGel: DMEM F-12), VitroGel-2 ratio 2:1, at day 3, day 7, day 11, and day 14. The figure illustrates the growth of kidney tubuloids over time and the changes between the tubuloids cultured with different hydrogels in morphology, number, and dimension.

Discussion

The organoids market is growing rapidly, and it is expected to have an annual growth of 21.7%, reaching 3.420 billion dollars in 2027 (Lee et al., 2021). Organoid culture plays a crucial role, and its development will continue to progress in tandem with the growth of the market. The aim of this project was to discover an animal-free matrix for culturing organoids, which would provide a more ethical and sustainable alternative to BME and Matrigel, which are the standards in our laboratory. The focus was on taking measured steps towards a more ethical and sustainable method of growing liver organoids and kidney tubuloids, with the potential to ultimately achieve better performance outcomes We firmly believe that this approach can become the new standard in the field, with even better performance outcomes. To achieve this objective, we conducted an experiment in which we tested three different animal-free matrix materials for their suitability in cultivating liver organoids and kidney tubuloids. We compared the performance of these materials to laboratory standards, Matrigel for liver organoids and BME for kidney tubuloids. However, despite the various combinations tested, none of the hydrogels were found to have the same performances as Matrigel and BME.

The evaluation of hydrogels for liver organoid cultures showed that different optimal ratios of dilutions exist for different versions of VitroGel 3D. Good results for organoid culturing were obtained with 100% VitroGel 1 and VitroGel 4 and with a 3:1 (VitroGel: DMEM F-12) ratio for VitroGel 1, VitroGel 2, and VitroGel 3. The optimal ratio for VitroGel 4 was 2:1 (VitroGel: DMEM F-12). The optimal combination was determined based on a comprehensive evaluation of all the parameters that were used to assess the comparison, which included the number of organoids and the rate at which they grow, their size, their morphology, and the results of their Rhodamine essay. The best results were obtained by considering all of these factors.

Of all the combinations tested, the best results were achieved with a 3:1 ratio of VitroGel 2. An interesting observation was made when the organoids were tested with Rhodamine assay, which revealed a tendency for the organoids cultured with VitroGel to form multiple lumens. However, the structural properties of the gel present a challenge, as it is unable to form domes like those cultured with Matrigel and may struggle to withstand the constant dynamic flow of the chip. In a study published in 2022 (Herpes et al., 2022), the formation of multiple lumens was observed in patient-derived colorectal cancer (CRC) organoids. The researchers hypothesized that the EGF and HRG pathways may have induced changes in morphology by altering the polarity and differentiation of tumor cells. The morphological changes observed in organoids can arise from various factors, and their effects on the overall viability and functionality of the organoids remain unclear. In accordance with our experimental findings, a recent study has reported that organoids cultured using VitroGel were found to be smaller in size compared to those cultured using Matrigel. Moreover, VitroGel has been successfully utilized as an animal-free matrix for gastric organoid culture and dendritic cells in a chip to simulate complex interactions in the gastrointestinal system (Cherne et al., 2021).

The results of liver organoid culturing using different dilution ratios of MaxGel in DMEM F-12 medium showed that the optimal ratio was 1:2 (MaxGel: DMEM F-12). The organoids cultured with MaxGel tend to have a spherical shape, similar to those obtained using Matrigel as the

culture matrix. The use of a non-adhesive plate was found to improve organoid growth and reduce the formation of a 2D monolayer, which can manifest if a normal cell culture plate was used. In terms of organoid properties, those obtained with MaxGel were found to be the most similar to those obtained with Matrigel. However, it is worth noting that the structural properties of MaxGel are comparable to those of VitroGel and therefore may not be suitable for use in a flow-based chip culture system.

MaxGel is a hydrogel derived from the co-culture of human fibroblast and epithelial cells *in vitro*, however, there have been other efforts to obtain hydrogels that are animal-free for organoid culturing. One potential approach involves the use of hydrogels derived from decellularized and solubilized organs, such as the liver. These types of hydrogels can be considered animal-free matrices since the organs utilized are human-derived and offer the advantage of being highly tissue-specific. Hydrogels derived from the liver have the same complex chemical components as the original organ, which allows for better replication of the microenvironment. Moreover, the chemical components of the hydrogel can be modified to mimic specific diseases, such as fibrosis. (Willemse et al., 2022). However, there are certain drawbacks that must be considered in utilizing this animal-free hydrogel organ derived. For instance, there could be an inadequate decellularizing process of the organ that could affect the organoid's culture (Morris et a., 2016). Additionally, the acquisition of healthy human livers for use in hydrogel preparation is challenging, and scaling up this process may also prove difficult. One potential solution to this issue is the use of cadaveric livers (Acun et al., 2021). Furthermore, there could be a batch-to-batch variability that could limit the reproducibility and potentially present similar issues as those encountered with Matrigel or BME (Hughes et al., 2010).

Different dilution ratios of PeptiGel Alfa 1 and PeptiGel Alfa 1 RGD on liver organoid culture showed different effects. Four different dilution ratios were employed and the ones with the best result were 1:2 (PeptiGel: DMEM F-12) for PeptiGel Alfa 1 and 1:1 (PeptiGel: DMEM F-12) for PeptiGel Alfa RGD. Observations revealed that the organoids displayed a rounded shape but showed limited growth potential confronted with the one cultured with Matrigel. The physical structure of the gel led to the formation of regions of varying density of the gel and cell concentration, which presented challenges for the maintenance of the culture. In comparison to other gels, PeptiGel was found to possess a unique property, as it was not a liquid but rather a gel-like substance. The dilution process with DMEM-F12 required caution to avoid the creation of bubbles, as it was performed by cutting the tip of a pipette and gently mixing. However, despite the efforts, the use of PeptiGel was found to be challenging, as the gel properties and formation of dense zones made it difficult to refresh the medium. Additionally, the results of the study revealed that the PeptiGel was not able to form domes, as observed with Matrigel, at any of the dilution ratios tested. Based on these findings, it is concluded that PeptiGel is not a suitable material for liver organoid culture and its use is not recommended.

In the process of splitting animal-free matrices, the companies that produce such matrices recommend using their own products. However, during my project, we sought to avoid purchasing new materials and instead tested multiple methods to identify the most efficient approach. Through experimentation, we found that cold DMEM F-12 can be used for the splitting of two hydrogels, excluding PeptiGel. This finding is significant because the splitting

process for these two hydrogels is similar to the standard protocol used for splitting Matrigel and BME.

In summary, additional experimentation is necessary to further evaluate the suitability of MaxGel and VitroGel for liver organoid culture. A quantitative PCR (qPCR) analysis should be conducted to assess the gene expression and if they can be indeed differentiated towards hepatocyte-like organoids. Additionally, an assessment of the mechanical properties of the hydrogels is necessary to determine their suitability for a microfluidic chip. This evaluation will provide crucial information regarding the potential for the integration of these animal-free hydrogels in a microfluidic platform.

The experimentation with an animal-free matrix for kidney tubuloids gave better results, the objective was to find a hydrogel that can be used to culture and maintain the tubuloids. The most favorable results were obtained with the VitroGel 2 ratio of dilution 2:1 (VitroGel: DMEM F-12) and the MaxGel ratio of dilution 1:2 (MaxGel: DMEM F-12). The organoids cultured with MaxGel showed the most promising characteristics, exhibiting a round shape and greater size compared to those cultured with VitroGel, though they are not as big as the ones cultured with BME. It is recommended to culture the kidney tubuloids in a suspension well plate, similar to the liver organoids. Although further experimentation, such as TEER measurement and qPCR analysis, is necessary, MaxGel may potentially be utilized in the future as an alternative to BME for kidney tubuloids culturing.

Limitations of this study include the number of liver organoids and kidney tubuloids that were very little, in fact, only the 96 well plates were used. Moreover, it is harder to see the mechanical properties of the gels compared to a 6-well plate. The hydrogels were tested with the cells with different passages, from 7^{th} to 13^{th}, which may have impacted the growth and the results. For future research, it is recommended to comprehensively test new gels broadly at various rates of dilution and under different conditions, as the instructions and suggestions provided by the manufacturers of animal-free hydrogels may not always yield optimal results. For instance, the suggested combination of animal-free hydrogels found in brochures has been found to be of limited use for organoid culture. While these combinations may initially seem promising, experimental evidence often does not support their effectiveness. The difference between the results from the company that made the product and the actual result may suggest that the performance of hydrogels may be specific to the type of organoid being cultured, as different types of cells may have varying requirements for optimal growth and function. Furthermore, this underscores the need for ongoing research to continue to develop and refine the use of animal-free hydrogels for organoid culture, as the optimal hydrogels for different types of cells and organs may not yet be fully understood or available on the market. However, it is important to approach the comparisons critically and prioritize finding a hydrogel that optimizes the growth and function of liver and kidney organoids, rather than just seeking similarity with the established gold standards.

Future Prospective

In the future, the adoption, and implementation of animal free-matrix for organoids culturing may lead to significant progress in their application, such as disease modeling the use of animal-free matrices can increase reproducibility and reduce variability, in contrast to animal-derived components. This approach offers the possibility of tailoring hydrogels to better imitate the microenvironment and hierarchical composition of native tissue in organoids (Maji et al., 2022). The hydrogels can be further customized for organoid-on-a-chip systems, making them more suitable for a constant fluid flow, and creating more accurate tissue models that are essential for predictive disease modeling (Skardal et al., 2017). It is possible that a comprehensive hydrogel database could be established, containing information on various hydrogel types and their specific features. This data could be analyzed using methods such as deep learning or artificial intelligence to assist in the development of hydrogels for specific cell groups or situations (Maji et al., 2022).

Conclusion

The objective of this project was to identify an animal-free matrix for liver organoids and kidney tubuloids, which would provide a more ethical and sustainable alternative compared to animal-derived matrices such as BME and Matrigel. One animal-free matrix appeared to be effective among the hydrogels tested for kidney tubuloids, the MaxGel, allowing the cells to grow and to be maintained for the period of the monitoring. Therefore, the use of VitroGel2 and MaxGel was shown to be the most effective for the cultivation of liver organoids, showing good results for the maintenance and for the Rhodamine essay. These results suggest that animal-free hydrogels can be a potential replacement for animal-derived matrix for organoids and tubuloids cultures. However, further experimentation is needed to validate the efficacy of these hydrogels to determine their optimal use in different situations. Moreover, novel animal-free matrices are being introduced into the market annually, and in the future researchers will have more options to culture organoids. However, it is important to approach the comparisons in a critical way and emphasize finding an animal-free hydrogel that optimizes the growth and function of liver organoids and kidney tubuloids, rather than just searching for products that give similar results as the established golden standards.

References

1. Acun, A., Oganesyan, R., Uygun, K., Yeh, H., Yarmush, M. L., & Uygun, B. E. (2021). Liver donor age affects hepatocyte function through age-dependent changes in decellularized liver matrix. *Biomaterials*, *270*, 120689. https://doi.org/10.1016/j.biomaterials.2021.120689
2. Aisenbrey, E. A., & Murphy, W. L. (2020). Synthetic alternatives to Matrigel. *Nature reviews. Materials*, *5*(7), 539–551. https://doi.org/10.1038/s41578-020-0199-8
3. Aloia, L., McKie, M. A., Vernaz, G., Cordero-Espinoza, L., Aleksieva, N., van den Ameele, J., Antonica, F., Font-Cunill, B., Raven, A., Aiese Cigliano, R., Belenguer, G., Mort, R. L., Brand, A. H., Zernicka-Goetz, M., Forbes, S. J., Miska, E. A., & Huch, M. (2019). Epigenetic remodelling licences adult cholangiocytes for organoid formation and liver regeneration. *Nature cell biology*, *21*(11), 1321–1333. https://doi.org/10.1038/s41556-019-0402-6
4. Ammirati A. L. (2020). Chronic Kidney Disease. *Revista da Associacao Medica Brasileira (1992)*, *66Suppl 1*(Suppl 1), s03–s09. https://doi.org/10.1590/1806-9282.66.S1.3
5. Broutier, L., Mastrogiovanni, G., Verstegen, M. M., Francies, H. E., Gavarró, L. M., Bradshaw, C. R., Allen, G. E., Arnes-Benito, R., Sidorova, O., Gaspersz, M. P., Georgakopoulos, N., Koo, B. K., Dietmann, S., Davies, S. E., Praseedom, R. K., Lieshout, R., IJzermans, J., Wigmore, S. J., Saeb-Parsy, K., Garnett, M. J., … Huch, M. (2017). Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. *Nature medicine*, *23*(12), 1424–1435. https://doi.org/10.1038/nm.4438
6. Cherne, M. D., Sidar, B., Sebrell, T. A., Sanchez, H. S., Heaton, K., Kassama, F. J., Roe, M. M., Gentry, A. B., Chang, C. B., Walk, S. T., Jutila, M., Wilking, J. N., & Bimczok, D. (2021). A Synthetic Hydrogel, VitroGel® ORGANOID-3, Improves Immune Cell-Epithelial Interactions in a Tissue Chip Co-Culture Model of Human Gastric Organoids and Dendritic Cells. *Frontiers in Pharmacology*, *12*. https://doi.org/10.3389/fphar.2021.707891
7. Chwalek, K., Bray, L. J., Werner, C., & Tissue Engineering Part B: Reviews, 20(6), 683-694.
8. Clevers, H. (2016). Modeling development and disease with organoids. Cell 165, 1586–1597. doi:10.1016/j.cell.2016.05.082
9. Clevers, H. (2016). Modeling development and disease with organoids. Cell, 165(7), 1586-1597.
10. Fatehullah, A., Tan, S. H., & Barker, N. (2016). Organoids as an in vitro model of human development and disease. Nature cell biology, 18(3), 246–254. https://doi.org/10.1038/ncb3312
11. Herpers, B., Eppink, B., James, M. I., Cortina, C., Cañellas-Socias, A., Boj, S. F., Hernando-Momblona, X., Glodzik, D., Roovers, R. C., van de Wetering, M., Bartelink-Clements, C., Zondag-van der Zande, V., Mateos, J. G., Yan, K., Salinaro, L., Basmeleh, A., Fatrai, S., Maussang, D., Lammerts van Bueren, J. J., Chicote, I., … Throsby, M. (2022). Functional patient-derived organoid screenings identify MCLA-158 as a therapeutic EGFR × LGR5 bispecific antibody with efficacy in epithelial tumors. Nature cancer, 3(4), 418–436. https://doi.org/10.1038/s43018-022-00359-0
12. Huch, M., Gehart, H., van Boxtel, R., Hamer, K., Blokzijl, F., Verstegen, M. M., Ellis, E., van Wenum, M., Fuchs, S. A., de Ligt, J., van de Wetering, M., Sasaki, N., Boers, S. J.,

Kemperman, H., de Jonge, J., Ijzermans, J. N., Nieuwenhuis, E. E., Hoekstra, R., Strom, S., Vries, R. R., … Clevers, H. (2015). Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell*, *160*(1-2), 299–312. https://doi.org/10.1016/j.cell.2014.11.050

13. Hughes, C. S., Postovit, L. M., & Lajoie, G. A. (2010). Matrigel: a complex protein mixture required for optimal growth of cell culture. *Proteomics*, *10*(9), 1886–1890. https://doi.org/10.1002/pmic.200900758
14. Jee, J. H., Lee, D. H., Ko, J., Hahn, S., Jeong, S. Y., Kim, H. K., Park, E., Choi, S. Y., Jeong, S., Lee, J. W., Cho, H. J., Kwon, M. S., & Yoo, J. (2019). Development of Collagen-Based 3D Matrix for Gastrointestinal Tract-Derived Organoid Culture. Stem cells international, 2019, 8472712. https://doi.org/10.1155/2019/8472712
15. Kleinman, H. K., & Martin, G. R. (2005). Matrigel: basement membrane matrix with biological activity. *Seminars in cancer biology*, *15*(5), 378–386. https://doi.org/10.1016/j.semcancer.2005.05.004
16. Kleinman, H. K., & Martin, G. R. (2005). Matrigel: basement membrane matrix with biological activity. Seminars in cancer biology, 15(5), 378–386. https://doi.org/10.1016/j.semcancer.2005.05.004
17. Lackner, C., Spindelboeck, W., Haybaeck, J., Douschan, P., Rainer, F., Terracciano, L., Haas, J., Berghold, A., Bataller, R., & Stauber, R. E. (2017). Histological parameters and alcohol abstinence determine long-term prognosis in patients with alcoholic liver disease. *Journal of hepatology*, *66*(3), 610–618. https://doi.org/10.1016/j.jhep.2016.11.011
18. Lancaster, M. A., Renner, M., Martin, C. A., Wenzel, D., Bicknell, L. S., Hurles, M. E., Homfray, T., Penninger, J. M., Jackson, A. P., & Knoblich, J. A. (2013). Cerebral organoids model human brain development and microcephaly. *Nature*, *501*(7467), 373–379. https://doi.org/10.1038/nature12517
19. Lee, H., Im, J. S., Choi, D. bin, & Woo, D.-H. (2021). Trends in the global organoid technology and industry: from organogenesis in a dish to the commercialization of organoids. *Organoid*, *1*, e11. https://doi.org/10.51335/ORGANOID.2021.1.E11
20. Liu, G., David, B. T., Trawczynski, M., and Fessler, R. G. (2020). Advances in pluripotent stem cells: History, mechanisms, technologies, and applications. Stem Cell Rev. Rep. 16, 3–32. doi:10.1007/s12015-019-09935-x trovare il giusto medoto di citazione
21. Liu, H., Wang, Y., Cui, K., Guo, Y., Zhang, X., & Qin, J. (2019). Advances in Hydrogels in Organoids and Organs-on-a-Chip. *Advanced materials (Deerfield Beach, Fla.)*, *31*(50), e1902042. https://doi.org/10.1002/adma.201902042
22. Michalopoulos, G. K., Bowen, W. C., Mulè, K., & Stolz, D. B. (2001). Histological organization in hepatocyte organoid cultures. *The American journal of pathology*, *159*(5), 1877–1887. https://doi.org/10.1016/S0002-9440(10)63034-9
23. Mokdad AA, Lopez AD, Shahraz S, Lozano R, Mokdad AH, Stanaway J, et al. Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. BMC Med 2014;12:145
24. Moon, A. M., Singal, A. G., & Tapper, E. B. (2020). Contemporary Epidemiology of Chronic Liver Disease and Cirrhosis. Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association, 18(12), 2650–2666. https://doi.org/10.1016/j.cgh.2019.07.060

25. Morris, A. H., Chang, J., & Kyriakides, T. R. (2016). Inadequate Processing of Decellularized Dermal Matrix Reduces Cell Viability In Vitro and Increases Apoptosis and Acute Inflammation In Vivo. *BioResearch open access*, *5*(1), 177–187. https://doi.org/10.1089/biores.2016.0021
26. Nguyen, V. V. T., Ye, S., Gkouzioti, V., van Wolferen, M. E., Yengej, F. Y., Melkert, D., Siti, S., de Jong, B., Besseling, P. J., Spee, B., van der Laan, L. J. W., Horland, R., Verhaar, M. C., & van Balkom, B. W. M. (2022). A human kidney and liver organoid-based multi-organ-on-a-chip model to study the therapeutic effects and biodistribution of mesenchymal stromal cell-derived extracellular vesicles. *Journal of extracellular vesicles*, *11*(11), e12280. https://doi.org/10.1002/jev2.12280
27. Nuciforo, S., & Heim, M. H. (2020). Organoids to model liver disease. *JHEP reports : innovation in hepatology*, *3*(1), 100198. https://doi.org/10.1016/j.jhepr.2020.100198
28. Ohnuki, M., & Takahashi, K. (2015). Present and future challenges of induced pluripotent stem cells. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *370*(1680), 20140367. https://doi.org/10.1098/rstb.2014.0367
29. Prior, N., Inacio, P., & Huch, M. (2019). Liver organoids: from basic research to therapeutic applications. *Gut*, *68*(12), 2228–2237. https://doi.org/10.1136/gutjnl-2019-319256
30. Protzer, U., Maini, M. K., & Knolle, P. A. (2012). Living in the liver: hepatic infections. *Nature reviews. Immunology*, *12*(3), 201–213. https://doi.org/10.1038/nri3169
31. Romagnani, P., Remuzzi, G., Glassock, R., Levin, A., Jager, K. J., Tonelli, M., Massy, Z., Wanner, C., & Anders, H. J. (2017). Chronic kidney disease. *Nature reviews. Disease primers*, *3*, 17088. https://doi.org/10.1038/nrdp.2017.88
32. Saorin, G., Caligiuri, I., & Rizzolio, F. (2022). Microfluidic organoids-on-a-chip: The future of human models. *Seminars in cell & developmental biology*, S1084-9521(22)00288-9. Advance online publication. https://doi.org/10.1016/j.semcdb.2022.10.001
33. Schutgens, F., & Clevers, H. (2020). Human Organoids: Tools for Understanding Biology and Treating Diseases. Annual review of pathology, 15, 211–234. https://doi.org/10.1146/annurev-pathmechdis-012419-032611
34. Schutgens, F.; Rookmaaker, M.B.; Margaritis, T.; Rios, A.; Ammerlaan, C.; Jansen, J.; Gijzen, L.; Vormann, M.; Vonk, A.; Viveen, M.; et al. Tubuloids derived from human adult kidney and urine for personalized disease modeling. Nat. Biotechnol. 2019, 37, 303–313
35. Sharma, A., & Nagalli, S. (2022). Chronic Liver Disease. In *StatPearls*. StatPearls Publishing.
36. Skardal, A., Murphy, S. V., Devarasetty, M., Mead, I., Kang, H. W., Seol, Y. J., Shrike Zhang, Y., Shin, S. R., Zhao, L., Aleman, J., Hall, A. R., Shupe, T. D., Kleensang, A., Dokmeci, M. R., Jin Lee, S., Jackson, J. D., Yoo, J. J., Hartung, T., Khademhosseini, A., Soker, S., … Atala, A. (2017). Multi-tissue interactions in an integrated three-tissue organ-on-a-chip platform. *Scientific reports*, *7*(1), 8837. https://doi.org/10.1038/s41598-017-08879
37. Stein, M. C., Braun, F., Krebs, C. F., & Bunders, M. J. (2021). Kidney organoid systems for studies of immune-mediated kidney diseases: challenges and opportunities. *Cell and tissue research*, *385*(2), 457–473. https://doi.org/10.1007/s00441-021-03499-4

38. Stepanova M, De Avila L, Af endy M, Younossi I, Pham H, Cable R, et al. Direct and indirect economic burden of chronic liver disease in the United States. Clin Gastroenterol Hepatol 2016
39. Tang, X. Y., Wu, S., Wang, D., Chu, C., Hong, Y., Tao, M., Hu, H., Xu, M., Guo, X., & Liu, Y. (2022). Human organoids in basic research and clinical applications. Signal transduction and targeted therapy, 7(1), 168. https://doi.org/10.1038/s41392-022-01024-9
40. Wieringa, P. A., Gonçalves de Pinho, A. R., Micera, S., van Wezel, R., & Moroni, L. (2018). Biomimetic Architectures for Peripheral Nerve Repair: A Review of Biofabrication Strategies. *Advanced healthcare materials*, *7*(8), e1701164. https://doi.org/10.1002/adhm.201701164
41. Willemse J, van der Laan LJW, de Jonge J, Verstegen MMA. Design by Nature: Emerging Applications of Native Liver Extracellular Matrix for Cholangiocyte Organoid-Based Regenerative Medicine. *Bioengineering*. 2022; 9(3):110. https://doi.org/10.3390/bioengineering9030110
42. Yousef Yengej FA, Jansen J, Rookmaaker MB, Verhaar MC, Clevers H. Kidney Organoids and Tubuloids. Cells. 2020; 9(6):1326. https://doi.org/10.3390/cells9061326