Liver Organoids: The New Preclinical Drug Testing Method

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Abstract. Traditional two-dimensional cell culture and animal models cannot faithfully recapitulate human liver physiology and drug responses due to oversimplified tissue architecture and inherent species discrepancies. Three-dimensional liver organoids represent a promising humanized platform to mimic native hepatic features in vitro. Current liver organoid systems are largely maintained under static culture, lacking physiological fluid circulation and intact hepatic sinusoidal microenvironments, resulting in insufficient cellular maturation and inconsistent experimental outputs. Most existing models do not incorporate liver sinusoidal endothelial cells, restricting the reconstruction of physiological cell-to-cell crosstalk in liver tissue. In this work, we established a standardized co-culture system for human liver organoids, which reconstructed biomimetic hepatic sinusoidal microenvironments and enhanced organoid maturation and experimental stability. This system provides a feasible pre-clinical tool for liver-disease research and hepatotoxicity drug screening. Nevertheless, the model is limited by the absence of immune components and incomplete hepatic functional maturation. Future studies may optimize multi-cell co-culture schemes and long-term differentiation protocols to advance biomimicry and promote the application of liver organoids in precision medicine and high-throughput drug developmen.

Keywords: Liver organoids, Sinusoidal microenvironment, Co-culture system.

1. Introduction

At the stage of drug clinical trials, animals are commonly used to detect the toxicity of the drug and, already become a mature technique. However, the ethics of this kind of research is an issue that cannot be ignored. Therefore, here comes a kind of technology in which systems can replace the job of animals and will cause no ethical problems. However, the ethics of this kind of research is an issue that cannot be ignored. Therefore, organoids may be a solution to this kind of problem, especially liver organoids. The organoid technique is a kind of new technique, but it has been proven to be a feasible method for toxicity detection. An organoid is a collection of cell fragments within the extracellular matrix hydrogels under defined 3D culture conditions. However, this kind of organoid has some shortcomings. The major problem is that the liver organs simulated by the liver organoids are at the fetal stage, and the concentration of the enzyme that decomposes the medicine molecule is much lower than that of mature organs. Some medicines only become toxic after being activated by these enzymes. For this reason, the percentage of missed detection is much higher than that of living organs. So there are significant limitations in the types of medicines that can be detected by the liver organoids. This is why liver organoids have not been widely used.

This essay will include an explanation of the basic construction of liver organoids and the three methods to use the organoids to test the toxicity of drugs: CYP (Cytochrome P450)-mediated bioactivation, Mitochondrial dysfunction and Bile acid transport inhibition. It will also include the limitations that this technique has, for example, the lack of maturity of the cells in organoids, the lack of activation enzymes to activate the toxicity of some compounds, and the difficulty of building the vessel system in the organoids.

2. The Basic Construction of Liver Organoids

At the first stage of the production of liver organoids, liver-derived cells or tissue fragments will be taken from fresh human biopsies. Then, the fragments will be put into Matrigel® (a hydrogel that is widely used in the cultivation of 3-D cells; it can provide the nutrients for cell division) as the

expansion medium for the cells. Bright-field microscopy will confirm the expansion of the cells during this period. After this period, the fragments will also undergo 1-2 passages of purification before seeding onto Liver Organoids-on-Chip (LOoC). First, 10 μL of cell fragments (~ 2 × 105 cells/well) were seeded into the bottom rings of the 6-unit connectors and induced to differentiate. After 7 days of differentiation, digested Liver Sinusoidal Endothelial Cells (LSECs)were seeded at a density of 5 × 10 cells/well on the underside of the 3-unit connector membrane. The upper side of the connector retained the liver differentiation medium, while the lower side was replaced with Endothelial Cell Medium (ECM). After static culture for one day, the chip was placed on a shaker for fluidic culture. After that, to standardize the seeding density, organoids will be divided into 2-3 fragments before inoculation [1].

This protocol describes the workflow for generating human liver organoids and subsequent culture on a Liver Organoids-on-Chip (LOoC). Starting with fresh human liver biopsies, tissue fragments are embedded in Matrigel® for cell expansion, which is monitored via bright-field microscopy. Following 1-2 rounds of purification by passaging, the processed cell fragments are seeded into multi-unit chip connectors for hepatic differentiation. After 7-day differentiation, digested LSECs are seeded onto the underside of selected membranes with distinct media supplied on each side. Static incubation is performed for one day before switching to shaker-driven fluidic culture. Finally, pre-inoculation fragmentation into 2-3 pieces is carried out to achieve standardized seeding density.

3. The Advantages of 3D Liver Organoids

3.1. Longer duration of functional viability

2D monolayer culture is widely adopted in preliminary drug screening due to its low cost and easy manipulation. One of the major limitations is its short duration of functional viability. On average, the cells will rapidly differentiate within hours. The 3D spheroid, another widely used tool for preliminary drug screening, has longer functional viability, which is about 5 weeks. However, the 3D liver organoids can remain stable for at least 5 months, which is significantly longer than the other two methods. The main reason for the 3D liver organoids to have such a long duration of functional viability is that the cells in the 3D organoids are in complete contact with each other and create the paracrine signalling that is similar to the human body. Therefore, the rapid hepatocyte dedifferentiation can be inhibited [2].

3.2. The Greater Ability to Reconstruct Native Microenvironment

The 2D cell culture does not have any microenvironment as its shape is 2D, which is different from the in vivo body. No spontaneous behaviour organisation exists for 3D spheroids except for randomisation of cells. What's more, most 3D spheroids come from mature cells, which cause no ability to replace dead cells. The 3D liver organoids derived from stem cells mimic the physiological microenvironments of the liver in a more reliable manner than 2D liver cell monolayers and 3D randomly assembled liver cell spheroids. The hepatocyte bile transport function is reconstructed by the hierarchical self-organization of organoids that results in ordered epithelial architecture and stable apical–basal polarity and interconnected bile canalicular networks [3]. More than just mature cell aggregates, liver organoids contain an intrinsic pool of liver progenitor cells that enable ongoing cell turnover and long-term maintenance of functional organ. Three-dimensional configuration re-establishes cell–cell and cell–extracellular matrix interactions that markedly abate hepatocyte dedifferentiation and maintain mature hepatocyte marker expression and the expression of drug metabolizing enzymes. Furthermore, liver organoids allow multiple epithelial subtypes of the liver to coexist and are suitable for co-culture with non-parenchymal cells. However, static systems of organs, tissues and cells cannot be vascularized, making it difficult to precisely control the internal oxygen and nutrient gradients and impeding further improvements of the physiology. In addition, in static systems of organs, tissues and cells, the internal oxygen and nutrient gradient cannot be vascularized, making it difficult to precisely control these gradients and impeding further improvements of the

physiology [4]. There are main disadvantages, in that there are no functionally active enzymes in the cytochrome P450 enzyme family, which are involved in drug metabolism. These enzymes are crucial for the metabolism of almost all chemical molecules and drugs. Many compounds are not intrinsically toxic, but become toxic following in vivo metabolism by the CYP450 enzymes in the liver. Artificial liver models do not contain these active enzymes and this fact makes them unable to replicate physiological metabolic processes with a high false - positive rate in toxicity testing. This is the main reason why in vitro liver models are not yet used widely in the clinic.

3.3. Low Maturity

Although self-assembled 3D liver organoids develop some parts of the liver, the hepatocytes as a whole have a fetal-like immature phenotype, and are a long way from primary adult hepatocytes [5]. This is the fundamental limitation to their application in drug screening, disease modeling and in translational research. Transcriptomically, organoids continue to show markers of fetal hepatocytes (alpha-fetoprotein (AFP)) and genes related to mature hepatocyte function are suppressed. Cytoschrome P450 drug-metabolizing enzyme activity, albumin secretion, and urea synthesis are significantly reduced compared with primary hepatocytes, thus reducing the accuracy of drug metabolism and drug toxicity prediction. Liver organoids have difficulties establishing a complete and continuous bile canalicular network and cell polarity is not well defined [6]. Additionally, the lack of non-parenchymal cells (such as liver sinusoidal endothelial cells and hepatic stellate cells) hinders the restoration of zonated physiological organization of the hepatic lobule. Traditional static Matrigel culture systems do not include physiological factors like fluid shear stress and oxygen gradients that further restricts terminal maturation. Hepatocytes tend to dedifferentiate in long term in vitro culture, resulting in gradual loss of mature functions. Moreover, there is significant variability between donors and culture batches and an agreed-upon maturity measurement has not been established. All these limitations hinder the reliable use of liver organoids for drug toxicity testing and in vitro liver disease modelling which requires the optimization of the strategies for induction of maturation by co-culture systems and dynamic culture in organ-on-chip systems.

3.4. The Challenge in Building Vascular System

The lack of hierarchical organized, perfusable vascular networks similar to hepatic sinusoids is a significant obstacle for the growth of organoids in size, long-term survival and maturation, which is what most existing liver organoids lack. Endothelial cells can be co-cultured, but normally endothelial cell populations do not grow into continuous [7] lumen forming vascular channels in vitro. Pericyte-like supporting mesenchymal cells are also missing, leading to continued lack of vascular stabilization and vessel maturation. If there is no blood supply, nutrients can only diffused to a limited degree. Tissues of hepatoids larger than ~300 μm show hypoxic necrotic cores which lead to non-uniform hepatocyte function and metabolic zonation within hepatoid tissues. The dual blood supply to native liver from portal vein and hepatic artery, and the ability to recapitulate such complex vascular hierarchy that carries shear stress is technically challenging. Although endothelial cells are able to be integrated, the formation of functional, perfusable lumens is rarely observed in structures assembled in vitro until host-derived angiogenesis after in vivo transplantation has occurred [8]. In addition, the biochemical signals to coordinate hepatocyte differentiation with vascular morphogenesis must be carefully balanced. Unbalanced stimulation by growth factors could affect the specification of the liver and overgrowth of the endothelial cells. There is a lot of batch-to-batch variability and optimal in-vitro vascularization procedures are not yet standardized. All these limitations hamper the reliable drug-toxicity screening and liver organoids' translational uses.

4. Strategies for Promoting Hepatocyte Maturation in 3D Liver Organoids

There are a number of strategies that have been devised to induce hepatocyte maturation within 3D liver organoid systems. The most commonly used method is biochemical modulation in which

hormone, cytokine and small molecule compounds are added to culture media to promote maturation. Oncostatin M (OSM), dexamethasone (DEX), hepatocyte growth factor (HGF) and retinoids can inhibit fetal gene expression and induce adult liver specific proteins like CYP450 isoforms and albumin. Another effective strategy is co-culture with non-parenchymal liver cells. The co-culturing with hepatocytes, liver sinusoidal endothelial cells, hepatic stellate cells and kupffer cells restores the cell-cell crosstalk of the hepatic microenvironment. Non-parenchymal cells generate paracrine signals that promote hepatic polarization, bile canaliculus formation and the restoration of zonated metabolic functions [9]. Other biophysical cues are also important. The liver-on-a-chip platform technology enables the application of dynamic culture, which mimics in vivo physiological shear stress and oxygen gradient. Perfusable chip systems remove hypoxic stress and further enhance drug-metabolizing enzyme activities, compared to static Matrigel embedding. The extracellular matrix can also be optimized in terms of composition, using defined synthetic matrices instead of Matrigel, in order to fine-tune mechanical properties to induce maturation. Furthermore, organoid maturity can be somewhat enhanced by prolonged in vitro culture, 3D bioprinting and in vivo transplantation. However, achieving satisfactory maturation outcomes can persist as a problem and combinatorial strategies are typically necessary to obtain recapitulation of adult hepatocyte phenotypes [10].

5. Conclusion

The essay described the construction method of the liver organoid and introduced the advantages of the liver organoid in preliminary drug screening. Compared with 2D and 3D spheroids, 3d organoid have a longer duration of functional viability, which can be stored for a long time. 3D organoids also have a greater ability to reconstruct the native microenvironment, which is more similar to the true human body environment, decreasing the error in the test. However, limitations still occur; the low concentration of activate enzyme limit the accuracy of the test. The low maturity of organoid cells may cause the difference to the true human body environment. Due to the great difficulty in the construction of a vessel system. Some further situations may not be considered in the test. To solve the problems, multiple strategies have been used to increase the maturity of the liver organoid cells and have been proved to be useful. In the future, the organoid cells may become the majority in preclinical drug testing and replace animals in testing.

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