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SPECIAL REPORT

Guidelines for Manufacturing and Application of Organoids: Liver

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Recent amendments to regulatory frameworks have placed a greater emphasis on the utilization of *in vitro* testing platforms for preclinical drug evaluations and toxicity assessments. This requires advanced tissue models capable of accurately replicating liver functions for drug efficacy and toxicity predictions. Liver organoids, derived from human cell sources, offer promise as a reliable platform for drug evaluation. However, there is a lack of standardized quality evaluation methods, which hinders their regulatory acceptance. This paper proposes comprehensive quality standards tailored for liver organoids, addressing cell source validation, organoid generation, and functional assessment. These guidelines aim to enhance reproducibility and accuracy in toxicity testing, thereby accelerating the adoption of organoids as a reliable alternative or complementary tool to animal testing in drug development. The quality standards include criteria for size, cellular composition, gene expression, and functional assays, thus ensuring a robust hepatotoxicity testing platform.

Keywords: Liver organoids, Quality standards, *In vitro* testing, Hepatotoxicity

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Introduction

In recent years, there has been a remarkable shift in the regulatory frameworks that govern non-clinical drug and chemical toxicity evaluations, which are used in making decisions utilizing *in vitro* testing platforms as an alternative to animal models (1, 2). Conventional animal model-based methods for assessing liver toxicity exhibit reduced reliability because of the biochemical metabolic differences between humans and animals (3). The transition, driven by ethical, scientific, and economic requirements, has resulted in a considerable demand for advanced tissue models that can emulate *in vivo*-like characteristics, thus facilitating more accurate predictions of drug efficacy and toxicity (4-6). Hepatotoxicity is the substantial risk that clinical trial participants are exposed to during drug development because of the vital roles of the liver in drug metabolism and detoxification processes (7). This often remains undetected during clinical trials or in the post-marketing stages, which emphasizes the urgency of addressing this challenge when considering toxicity across various organs. To address this challenge, researchers have made substantial advancements in developing mature liver models that closely exhibit *in vivo* conditions, including liver organoids (8, 9).

Liver organoids have been widely adopted for high-throughput hepatotoxicity assessment (7-14). Organoids are human stem cell-based *in vitro* tissue models that recapitulate the complex structure, function, and cellular variations of human tissue. Liver organoids are generated from human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cell (iPSCs), or liver tissue-derived human adult stem cells (hASCs) (8, 9, 13, 15, 16). Their ability to mimic biological variations and organ-specific functions has made liver organoids a valuable tool for predicting *in vivo* hepatotoxicity during non-clinical trials. The liver's intricate architecture comprises liver lobules and essential vascular networks that support its functionality. Recent progress in generating matured liver organoids, characterized by multi-lineage incorporation and vascularization, has significantly advanced the replication of the liver's structural complexity and intercellular communication, enhancing its overall functionality (17, 18). Notably, the structural integrity within the functional luminal vasculature within these organoids closely resembles *in vivo* conditions, facilitating more accurate simulations of the drug, oxygen, and nutrient transports (17). This fidelity to physiological conditions holds promise for improving predictions in toxicity testing and therapeutic efficacy assessments.

The use of organoid models to evaluate liver toxicity presents several practical advantages, primarily in their enhanced accuracy in replicating human physiological systems using human cell sources. Additionally, these models enable long-term maintenance, which is a critical factor for investigating the chronic effects of toxic substances. Despite these benefits, there is a notable gap in the discussion regarding the necessary quality evaluation for standardizing organoids in the context of organoid-based toxicity testing (4). It is crucial to establish comprehensive guidelines to assess the functional quality of liver organoids to ensure the reproducibility and high accuracy of these toxicity testing platforms (19).

In this report, we propose comprehensive quality standards designed specifically for toxicity testing within the designated context of use (COU) to provide three major guidelines. First, reproducible culture conditions for liver organoids are defined. Second, the required characteristics of the cells from which liver organoids originate are clarified. Third, the quality assessments for the generated liver organoids are outlined across the morphological, gene expression, and functional levels. The development of these protocols and the proposal of these quality guidelines aim to provide standardized quality assessment criteria, thereby enhancing the reliability and acceptance of liver organoids in a regulatory context. By implementing these standardized guidelines, it is expected that the adoption of liver organoids as a platform for toxicity testing, will be accelerated, which will lead to a reduction in reliance on animal testing and support in the development of safer and more efficient pharmaceuticals and chemicals.

Materials and Methods

Cell cultures and reagents

Each step in the cell and organoid cultures strictly adheres to aseptic techniques. Culture conditions are maintained at a temperature of 37℃ and in a 5% CO_2 environment. Please refer to Supplementary Table S1 for comprehensive details on each reagent.

Liver organoid generation from hASCs: The isolation of primary hepatocytes typically follows established protocols (16). Following the isolation from patient liver samples, primary hepatocytes are initially washed with advanced Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) and centrifuged at 300～400 ×*g* for 5 minutes to pellet the cells. These cell pellets are then combined with Matrigel^{TM} (Corning) and seeded at a density of 3,000～10,000 cells/well in a 48-well plate. Once the cells are seeded, the Matrigel is allowed to solidify, at which point starting media (SM) (18) is added. This SM

is a blend of expansion media (20) supplemented with 25 ng/mL Noggin, 10 μM Y27632, and 100 ng/mL Wnt-3a. The composition of the expansion media (20) includes advanced DMEM/F12 supplemented with 1% penicillin-streptomycin (PS), 1x N-2, 1X B-27, 50 ng/mL epidermal growth factor (EGF), 25 ng/mL hepatocyte growth factor (HGF), 5 μM A83-01, 10 μM forskolin, 1.25 mM N-acetyl L-cysteine, 10 mM nicotinamide, 10 nM Leu15-gastrin I human, 10 ng/mL fibroblast growth factor 10 (FGF10), and 1 mg/mL R-spondin 1. The cells are maintained in the SM for three days. On the third day of culture in SM, the medium is replaced with expansion media, and after a further 10～14 days, the organoids are resuspended in freshly mixed Matrigel. For long-term maintenance, the resuspension can be repeated every 7～10 days for at least six months, with a dilution ratio ranging from 1 : 4 to 1 : 8 as appropriate.

Liver organoid differentiation from hPSCs: hPSCs, including both iPSCs and ESCs, undergo differentiation that mirrors the stages of liver development. Detailed steps for this process are provided in a previous study (8). Liver organoids derived from hPSCs are maintained and cultured using growth medium (GM), which consists of advanced DMEM/F12 supplemented with 1% PS, 1 mM GlutaMax, 1 mM HEPES, 1X N-2, 1X B-27 without vitamin A, 1X ITS, 50 ng/mL EGF, 25 ng/mL HGF, 10 ng/mL FGF-basic, 10 ng/mL Oncostatin M, 5 μM A83-01, 10 μM forskolin, 1 mM N-acetyl L-cysteine, 10 nM Leu15-gastrin I human, 10 mM nicotinamide, and 100 nM dexamethasone. For expansion, organoids cultured in a Matrigel dome and sustained with GM. The GM medium is replenished every three days. Regarding splitting, the organoids are isolated from the Matrigel matrix and can be divided either physically or chemically. Splitting is typically performed weekly, and the splitting ratio is adjusted between 1 : 4 and 1 : 10 according to the growth status. For differentiation, the cell medium is replaced with fresh EM a day after splitting. The composition of EM for the PSC-driven organoids differs slightly from the medium used for the ACS-driven organoid protocols. The EM consists of advanced DMEM/F12 supplemented with 1% PS, 1 mM GlutaMax, 1 mM HEPES, 1x N-2, 1X B-27 without vitamin A, 50 ng/mL EGF, 25 ng/mL HGF, 100 ng/mL FGF10, 1 mg/mL R-spondin 1, 25 ng/mL BMP7, 5 μM A83-01, 10 μM forskolin, 1 mM N-acetyl L-cysteine, 10 mM nicotinamide, and 10 nM Leu15-gastrin I human. Subsequently, the organoids are maintained in the EM for three days. Following this, the medium is exchanged with differentiation medium (DM) (8) and the cultures are maintained in DM for six days. Throughout this period, the DM medium is refreshed with fresh DM every two days. Upon completion of the differentiation process, the organoids are characterized and utilized for the subsequent toxicity assessments.

Quality assessment metrics

Gene expression: Total RNA is firstly extracted from the sample using RNA extraction reagents (easy-BLUE; iNtRON). The isolated RNA is subsequently converted into complementary DNA through reverse transcription polymerase chain reaction (RT-PCR) using TOPscript™ RT DryMIX (Enzynomics). Specific primers (Supplementary Table S2) that are designed for the gene-specific markers are utilized to quantify the expression levels through real-time PCR using Fast SYBR® Green Master Mix (Applied Biosystems).

Flow cytometry: The organoids are dissociated into single cells using enzymes such as TrypLE™ Express Enzyme (Gibco) for 10 minutes at 37℃. Subsequently, the single cells are then fixed, permeabilized, and sequentially blocked according to standard immunostaining protocols. Staining with an albumin (ALB)-specific antibody is then conducted, followed by flow cytometry analysis to assess the composition of ALB-positive cells within the liver organoids.

ALB, α1-antitrypsin, and urea secretion: To quantify the ALB, α1-antitrypsin (AAT), and urea levels, samples of the medium are collected 48-hour post-medium change and analyzed using a Human Albumin enzyme-linked immunosorbent assay (ELISA) Kit (Bethyl Laboratories), Human Alpha-1-Antitrypsin ELISA Quantitation Kit (Genway Biotech), or Urea Assay Kit (Cell Biolabs) according to the manufacturer's protocols. The absorbance readings are measured using a microplate reader and normalized by the cell count.

Activation of drug-metabolizing enzyme: To assess cytochrome P450 (CYP) activity, the organoids are induced with specific inducers (20 μM rifampicin for CYP3A4) for 48 hours. Following this induction, the CYP enzyme activities are assessed using subtype-specific substrates and a P450-Glo Assay Kit (Promega) in accordance with the manufacturer's protocols. Luciferase activity can be measured with a luminometer and normalized by the cell count.

Immunostaining: The organoids are initially fixed using 4% paraformaldehyde (Biosesang), followed by permeabilization of the cells using Triton X-100 at 21℃～23℃, and subsequent blocking. Liver cell-specific antibodies (Supplemenary Table S3) are applied for immunostaining, following the protocol that are used in the flow cytometry for ALB detection. Fluorescent microscopy is used to capture images of the stained organoids.

Periodic acid-Schiff staining: To investigate the storage of glycogen, we performed a series of procedures on the organoids. Frozen sections are cut to a thickness of 10 μm

at 20℃ using a cryostat microtome and treated with a periodic acid solution and then with Schiff's reagent using a periodic acid-Schiff (PAS) staining kit (IHC WORLD). Subsequently, the nuclei are stained using hematoxylin solution according to the provided instructions. The images are captured by optical microscopy.

Indocyanine green staining: For the analysis of indocyanine green (ICG) uptake and release, organoids are washed with cold phosphate-buffered saline (PBS) (Welgene) to remove the Matrigel and then incubated with ICG (Sigma-Aldrich) for 15 minutes at 37℃ in a 5% CO_2 atmosphere. Microscopic images are captured to observe ICG uptake. Subsequently, the organoids undergo three gentle washes with PBS and fresh medium is added. After incubation for an hour at 37℃ in 5% CO_2, images are taken to observe ICG release.

Carboxyfluorescein diacetate staining: For the functional polarization assay, the organoids are detached from the Matrigel and incubated with culture media supplemented with carboxyfluorescein diacetate (CDFDA; Sigma-Aldrich) and Hoechst 33342 (Thermo Scientific) for 30 minutes at 37℃ in a 5% CO_2 atmosphere. After two gentle washes with cold PBS containing calcium and magnesium (Gibco), fluorescence images are captured using a confocal microscope.

Liquid chromatography-tandem mass spectrometry: Coupling of liquid chromatography-tandem mass spectrometry (LC-MS/MS) with chemical isotope labeling or isotope-labelled standard enables the quantitative examination of changes in the expression levels of targeted proteins and metabolites in liver organoids. The prerequisite sample preparations for the LC-MS experiments can be sequentially conducted–protein extraction, proteolytic digestion and chemical isotope labeling. If specific proteins are defined for quality control of liver organoids, add isotope-labeled counterpart peptides to each sample of liver organoids and their respective controls to be in suit with the mole quantities of the targeted proteins. In this paper, there are no restrictions on the types of instruments for LC-MS/MS experiments, excepting data analysis of MS/MS spectra obtained by LC-MS/MS. To enhance reliability of quantitative determination, there must be minimum requirements of search criteria as follows: (i) both software and proteome database must be validated, and (ii) protein searching against protein database are carried out with strict criteria as two missed cleavages per peptides, mass tolerance for precursors and fragment ions, fixed/variable modifications on amino acid residues of protein, the false discovery rate of <0.01 at both peptide and protein levels. The isotopic ratios of targeted proteins to controls are transformed into log2 scale and normalized by median subtraction. This allows for the quantitative comparison of changes in the expression levels of targeted proteins derived from liver organoids compared to the control groups (such as primary human hepatocytes, PHHs). There are no restrictions on the protein extraction methods, enzyme for proteolytic digestion, stable isotope labeling techniques, or LC-MS/MS configuration.

Protocols for storage

Freezing process: For long-term storage, liver organoids can be preserved by freezing. After separating the organoids from the Matrigel dome during cultivation, organoid pieces are obtained via physical or chemical dissociation. The remaining Matrigel and debris are thoroughly removed using basal medium (BM). The organoid pieces are suspended in a freezing solution such as mFreSR (STEMCELL Technologies) and transferred to cryotubes. They are placed in a cryotank and stored in a deep freezer for 24 hours before being transferred to a liquid nitrogen (LN_2) tank for long-term storage.

Thawing process: Organoid stock vials that were frozen in LN_2 are removed and rapidly thawed to approximately 50% in a 37℃ water bath. BM+Y-27632 (Tocris) is added, and the stock solution is dissolved via pipetting. After sedimenting the organoid pieces using a centrifuge, they are washed 1～2 times with BM. The washed organoid pieces are mixed with Matrigel and domed, and cultured with GM+Y-27632. The following day, the medium is replaced with fresh GM+Y-27632, and cultured for an additional 2 days. On the third day, the medium is replaced with fresh GM and cultured.

Results

Quality standards for cell sources

General requirements for the cell source: Liver organoids can be generated from human liver tissue-derived ASCs, or PSCs, including ESCs and iPSCs. When sourcing cells for organoid generation from human donors, it is essential to comply with the ethical guidelines and obtain approval from the institutional review board (IRB) to ensure compliance with ethical considerations.

Quality management: Before starting the production of organoids, a thorough assessment of the original cell sources is required. This involves examining whether the cells are capable of surviving and proliferating under the intended culture conditions. In addition, it is crucial to conduct rigorous checks for any contamination that may compromise the integrity of the cell culture, thus ensuring that

the resulting organoids are of high quality and free from external pollutants. Furthermore, purification processes must be performed on hASCs to isolate and enrich the desired cell populations. This purification step enhances the consistency and reliability of the organoid culture by minimizing the presence of unwanted cell types. Another essential step of the preproduction process is validating the characteristics of the origin cells. This includes confirming that the cells exhibit the desired traits and functions relevant to their intended use in organoid development. These validations may include assessing specific molecular markers, determining cellular morphology, and performing functional assays to ensure that the cells possess the necessary properties for organoid formation and function. Furthermore, ensuring genetic stability is crucial for preventing any undesirable genetic alterations that may arise during cell culture and manipulation. Comprehensive genetic analyses should be conducted to verify the genomic integrity of the cells, thereby safeguarding against potential genetic abnormalities that could compromise the authenticity and functionality of the resulting organoids.

Cell characteristics and quality requirements of hASCs: For the generation of hASC-derived liver organoids, cells are sourced from PHHs obtained via biopsy of human clinical specimens (Fig. 1). The source cells for the generation of hASC-derived liver organoids exhibit stem cell and liver progenitor markers, such as LGR5, CK19, and SOX9. In particular, EpCAM+ ductal cells are sorted, and organoids are generated in three-dimensional (3D) through the addition of extracellular matrix and liver-specific growth factors. EpCAM+ cells possess bipotent characteristics, which enable them to differentiate into mature parenchymal cells in the liver, including cholangiocytes and hepatocytes (16, 21). Additionally, they must possess a normal karyotype, and remain uncontaminated by mycoplasma.

Cell characteristics and quality requirements of hPSCs: For the generation of hPSC-derived liver organoids, iPSC cells are primarily sourced, while ESC-derived liver organoids are also required the same quality assessment standards (Fig. 1). iPSCs are readily available for procurement through commercial channels, such as stem cell banking systems, at either the national administration or industry levels. Additionally, iPSCs can be generated in-house through the process of reprogramming somatic cells, including skin fibroblasts, blood cells, bone marrow cells, and urine-derived somatic cells. This reprogramming entails the introduction of Yamanaka factors (22), a set of specific transcription factors, into the somatic cell types that induce them to revert to a pluripotent state resembling that of ESCs. iPSCs that are used in the production of liver organoids should express pluripotency markers including NANOG, SOX2, and OCT4. They should also demonstrate the capacity for differentiation into all three germ layers, possess a normal karyotype, and be confirmed free from contamination by mycoplasma.

Quality requirements for liver organoids

In this section, we explore the essential factors involved

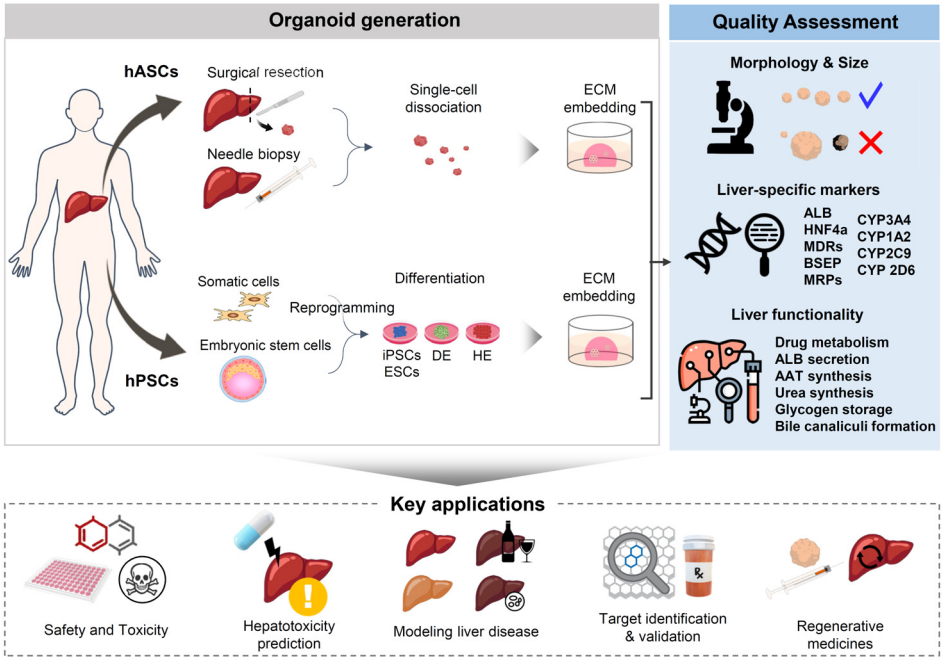

Fig. 1. A schematic diagram of liver organoid generation, quality assessment, and applications. To unlock the potential of liver organoids for diverse biomedical applications, it is essential to standardize the processes for generating organoids and assess their quality in terms of liver-like phenotypes and functionality. hASCs: human adult stem cells, hPSCs: human pluripotent stem cells, iPSCs: induced pluripotent stem cell, ESCs: embryonic stem cells, DE: definitive endoderm, HE: hepatic endoderm, ECM: extracellular matrix, ALB: albumin, AAT: α1-antitrypsin.

in the generation of liver organoids derived from hASCs or hPSCs, considering the quality standards regarding their size, morphology, and cellular composition. In addition, we offer guidelines for quality assessment to validate the organ-specific functions of the liver organoids. These guidelines evaluate both in qualitative and quantitative criteria.

Size, morphology, and cellular composition quality guidelines for liver organoids: hASC-derived liver organoids exhibit a spherical shape (Fig. 2A). While there are no strict size requirements, researchers should aim for a minimum size that ensures functional relevance. The ASC-derived liver organoids primarily consist of two key cell types: hepatocytes and cholangiocytes.

hPSC-derived liver organoids offer greater flexibility in terms of size and complexity because of their pluripotent nature. Researchers should define both the minimum and maximum size requirements for consistency. The morphology of iPSC-derived organoids under growth conditions typically appears spherical (Fig. 2B, left), while under differentiation conditions, they may include specific structures such as bile ducts or vessel-like networks (Fig. 2B, right), which enhances their physiological relevance (18, 20). Their cellular compositions may include hepatocytes, cholangiocytes, hepatic stellate cells, sinusoidal endothelial cells, and immune cells (23-25).

Quality requirements for liver-specific functions of the organoids: The capacity to replicate major liver functions is one of the primary requirements for the quality assessment of liver organoids. The fundamental functions to be validated include the secretion of the essential proteins ALB and AAT. In addition, liver organoids exhibit the ability to produce urea, a waste product of protein metabolism, and the ability to store glycogen. The absorption and clearance of ICG validate the critical liver functions in the detoxification process of liver organoids. Moreover, the dynamic bile production and excretion, activation of drug-metabolizing enzymes, detoxification (or metabolism) of xenobiotics, including pharmaceutical compounds, should be assessed to validate the liver-specific functions of the organoids.

Genomic integrity and identity: Liver organoids produced for toxicity testing should exhibit a normal chromosomal karyotype. The source cells (liver tissue or iPSCs) should display the same short tandem repeat. For the long-term maintenance of the liver organoids, the chromosomal karyotype is recommended to be analyzed every 20～30 passages.

Quality assessment for liver organoids

For the practical utilization of liver organoids as a hepatotoxicity testing platform, it is required to have a set of minimum standards for their quality, including their size, targeted cell numbers per size, cell compositions, gene expression, and functional assay. Based on our previous experiences in producing liver organoids for use as a toxicity testing platform, we propose the following quality standards for each endpoint.

Liver organoid size standards: For hepatotoxicity testing, it is recommended to utilize hASC-derived liver organoids with a minimum size of 100 μm and an average cell count exceeding 1,000 cells/organoid. hPSC-derived liver organoids should fall within the diameter range of 100～500 μm, with a prerequisite of more than 1,000 cells/organoid. The maximum size limit for hPSC-derived liver organoids is suggested because of potential limitations in nutrient and oxygen perfusion, which may lead to necrosis

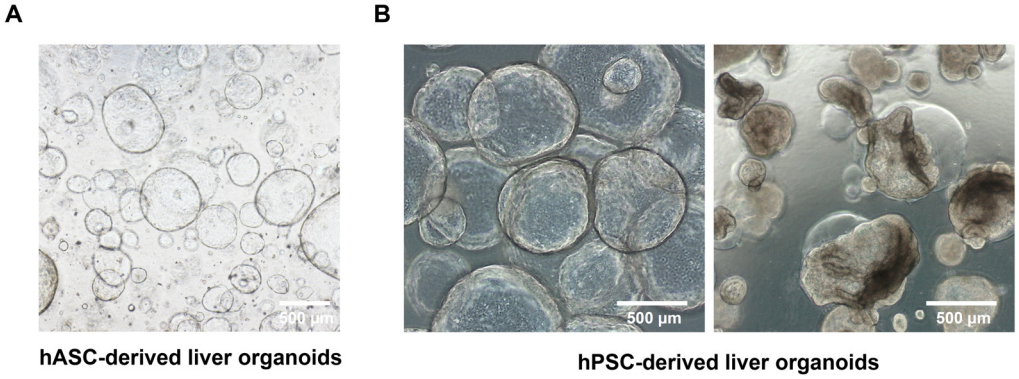

Fig. 2. Representative morphologies of liver organoids derived from (A) human adult stem cells (hASCs) and (B) human pluripotent stem cells (hPSCs). (A) Micrograph of the hASC-derived liver organoids present a spherical morphology. (B) hPSC-derived liver organoids under growth conditions display a spherical feature (left), while their morphologies undergo transformation during differentiation conditions (right). Scale bars = 500 μm.

in the central regions. Conversely, hASC-derived liver organoids, with their inherent cystic morphology, do not exhibit such issues.

Liver organoid cell composition requirements: Given the primary objective of liver organoids as platform for hepatotoxicity evaluation, it is important that their cellular composition predominantly reflects that of hepatocytes. Quantitatively assessing the proportion of hepatocytes within the liver organoids is determined by measuring the expression level of ALB using flow cytometry. Because human liver tissue typically consists of >70% hepatocytes (26, 27), it is recommended to utilize organoids that express ALB at levels >70%.

Gene expression and functional assays: Liver organoids are required to express liver-specific functional markers, including ALB, HNF4a, MDRs, BSEP, MRPs, and major drug-metabolizing enzymes, including CYP3A4, CYP1A2, CYP2C9, and CYP2D6, at both the RNA and protein levels. The liver-specific gene expression is quantitatively assessed using quantitative RT-PCR, followed by the detection of the protein levels in the supernatant of the organoid culture, which potentially reflects the liver functionality of the organoids. Liver organoids synthesize and secrete serum proteins, such as ALB and AAT, which are detected using ELISA. Alternatively, relative quantitative analysis can be performed to assess hepatocyte function using LC-MS/MS. This method enables the quantitative assessment of ALB protein levels, which can be compared with controls, such as PHHs. Moreover, the activity of drug-metabolizing enzymes can be measured after induced by the drug. The ammonia removal process generated through amino acid metabolism, which is one of the important liver functions, is observed through the production of urea. Additionally, liver organoids accumulate glycogen, absorb and excrete ICG, and have the ability to excrete bile, all of which can be qualitatively assessed using PAS, ICG, and CDFDA staining, respectively.

The quality assessment criteria for the above functions are evaluated quantitatively or qualitatively, as described in Table 1 and 2. The quantitative criteria were determined by referring to previously reported PHH data (8). For the specific COU for hepatotoxicity testing, it is recommended to use liver organoids that fulfill the quantitative evaluation criteria for at least the following liver-specific functions: (i) the cellular composition for ALB expression; and (ii) CYP3A4 activity. These functions serve as fundamental and essential standard indicators of liver functionality for drug metabolism.

Table 1. Quantitative criteria for the assessment of liver organoids

Endpoint	Method	Evaluation criteria
Liver-specific gene expressions	PCR	*ALB, HNF4a, MDRs, BSEP, MRPs, CYP3A4, CYP1A2, CYP2C9, CYP2D6,* etc.
Liver cell compositions	Flow cytometry	[a]>70%, cells expressing ALB
Activity of drug-metabolizing enzyme	CYP3A4 activity	[a]>1.5 fold, with rifampicin induction after treatment for 48 hours
ALB synthesis	ELISA or LC-MS/MS	>1 μg/24 h/10^6 cells, ALB secretion level
AAT synthesis	ELISA or LC-MS/MS	>1 μg/24 h/10^6 cells, AAT secretion level
Urea synthesis	Urea assay or LC-MS/MS	>1 μg/24 h/10^6 cells, urea secretion level

ALB: albumin, AAT: α1-antitrypsin, PCR: polymerase chain reaction, CYP: cytochromeP450, ELISA: enzyme-linked immunosorbent assay, LC-MS/MS: liquid chromatography-tandem mass spectrometry.
[a]Minimum criteria for the quality assessment of liver organoids.

Table 2. Qualitative criteria for the assessment of liver organoids

Endpoint	Method	Evaluation criteria
Liver-specific markers	Immunostaining	Positive staining for ALB, HNF4a, MDRs, MRPs, etc.
	PCR	Increased gene expression of *CYP3A4, CYP1A2, CYP2C9,* etc., following treatment with inducer drugs
Glycogen storage	PAS staining	Positive staining for PAS
ICG	ICG staining	Uptake and release of ICG
Bile canaliculi	CDFDA staining	Bile canaliculi structure observed after CDFDA staining

ICG: indocyanine green, PCR: polymerase chain reaction, PAS: periodic acid-Schiff, ICG: indocyanine green, CDFDA: carboxyfluorescein diacetate.

Monitoring of quality assessment results (batch-wise, periodic analysis, etc.)

Among the quality quantification criteria (Table 1), it is recommended to perform batch-wise toxicity evaluations for ALB secretion and CYP3A4 activity. Other quantitative and qualitative quality evaluations should be performed for all parameters after the initial production of organoids, and thereafter, monitoring every 10～20 passages is recommended.

Quality control for storage and preservation

Quality assurance in the storage and preservation of organoids depends on two key factors: post-thaw viability and the absence of microbial contamination. Organoids are cryopreserved for extended periods, which requires careful handling during both storage and subsequent post-thawing. It is crucial to ensure that organoids retain a viability threshold of >70% upon thawing; failure to meet this standard warrants the consideration of using an alternative batch from the frozen stocks. Furthermore, sustained, long-term maintenance requires rigorous measures to prevent microbial contamination. The detection of mycoplasma, a common contaminant, is achieved via PCR analysis using the supernatant medium from the organoid cultures following 48 hours of incubation. Negative PCR results, compared to the positive controls, indicate the absence of mycoplasma contamination. It is recommended to perform the contamination assessment every 10～20 passages to maintain the integrity of the organoid cultures.

Ethical considerations

Liver organoids must be produced in accordance with ethical and medical guidelines. Here, we outline specific criteria for selecting liver samples to create organoids derived from tissue-derived ASCs. Human liver tissue sampling should only be conducted from donors who willingly consent to participate in the research, particularly among patients who require surgical treatment. Donors over the age of 80, experiencing bleeding in the surgical region, not requiring surgical treatment, or under the age of 5 years old should be excluded. Sampling of liver tissue from patients with liver cancer or other liver diseases should be performed on the removed liver tissue from their surgical treatment. For other donors, tissue sampling may involve a portion of the tissue biopsy obtained for diagnosis. The personal information of any patient is codified and managed using clinical trial numbers, which are encrypted for protection. Encrypted information can only be accessed by the research participants, and disclosure to third parties is prohibited. When recruiting participants, medical staff must thoroughly explain that participation in the study will not result in any disadvantages or discriminatory treatment for the patient, and efforts should be made to ensure the autonomy and protection of vulnerable subjects. Patient consent, sample acquisition, and all other aspects of organoid research must be conducted under IRB approval.

Discussion

In this report, we offer critical considerations for the quality assessment of liver organoids to ensure their suitability for subsequent applications. This report outlined the prerequisites and assessments involved in sourcing cells, validating their characteristics, and evaluating the quality of liver organoids. The quality management protocols aim to enhance organoid consistency, confirm their suitability as a liver model, and ensure their fidelity and reliability as a testing platform, while adhering to strict ethical compliance.

These suggested guidelines for liver organoid quality assessment include rigorous evaluation of organoid morphology, functionality, and reliability of *in vivo* liver tissue characteristics. Various imaging modalities, biochemical assays, and functional tests are used to determine the structural integrity, metabolic activity, and responsiveness of the organoids, thereby providing insights into their suitability for hepatotoxicity testing. Criteria for hepatotoxicity testing platforms include size specifications, cell composition predominance, gene expression profiling, and functional assays, with specific quantified and qualified standards for liver-specific characteristics. Integration of these quality standards ensures the reliability and relevance of the liver organoids for toxicity testing applications.

Additionally, we highlight the significance of meticulous sourcing of cells, recognizing that the quality of organoids fundamentally relies on the initial quality of the cells used for their construction. This involves careful consideration of the quality control of the source cells, considering factors such as genetic stability, identity, and purity, to establish confidence in their reliability for organoid generation. Moreover, the procurement or sampling processes should adhere to strict ethical guidelines and regulatory standards to ensure the integrity and legitimacy of the cell source. Ethical compliance and approval from the IRB are essential when sourcing cells from human donors or patients for the generation of liver organoids.

This report provides essential insights into the quality assessment of liver organoids, ensuring their appropriateness for future applications as described in Fig. 1. As organoid technology advances toward better mimicking *in vivo* liver

functions and conditions, liver organoids are being considered as potential alternatives to animal testing platforms for assessing the safety and toxicity of chemicals (1, 4). They are also regarded as valuable *in vitro* platforms for innovating drug development, particularly in predicting hepatotoxicity during the early stages of drug development (12). Furthermore, liver organoids serve as versatile models for various liver diseases, enabling in-depth investigation of pathological processes and potentially expediting target identification and validation processes under conditions closely resembling those of human organs (9). Eventually, efforts to develop mature organoid models hold great promise for regenerative medicine (28). In this matter, our suggestions to establish standards for the generation processes and quality assessment of liver organoids are anticipated to significantly enhance the development of practical applications, particularly in hepatotoxicity testing.

Quality assessments need to be developed as a simple and reproducible analysis of the quality of organoids and sourced cells, to overcome the shortcomings of existing experimental validation studies. Recently, research has been performed in various fields to standardize organoids. In particular, an RNA-seq-based organ-specific gene expression panel was developed using the GTEx public database (29, 30). This system allows for the quantitative analysis of similarity to the target organ. Facilitating direct comparisons of the similarity and gene expression patterns between the target human organs and organoids improves organoid quality. Moreover, artificial intelligence- based image analysis provides sophisticated and accurate results in place of experiment-dependent morphology and functional feature analysis (31). The combination of high-throughput screening technology, high-capacity imaging systems, and machine-learning algorithms will enable the characterization of organoids in a simple and reproducible manner. These innovative approaches have the potential to solve these key challenges related to large-scale 3D culture and toxicity evaluation using liver organoids.

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Potential Conflict of Interest

There is no potential conflict of interest to declare.

Authors' Contribution

Conceptualization: MJS, SJA, DC. Data curation: HRM, SJM, THK. Formal analysis: HRM, SJM, THK. Funding acquisition: SJA, MJS. Investigation: HRM, SJM, THK. Methodology: SJM, THK, JHS. Project administration: MJS, SJA, DC. Resources: SJA, MJS. Supervision: MJS, SJA, DC. Validation: HK, DK, SK. Visualization: HRM, SJM. Writing – original draft: HRM, SJM. Writing – review and editing: HRM, MJS.

Supplementary Materials

Supplementary data including three tables can be found with this article online at https://doi.org/10.15283/ijsc24044

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