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REVIEW ARTICLE

Advanced Animal Replacement Testing Strategies Using Stem Cell and Organoids

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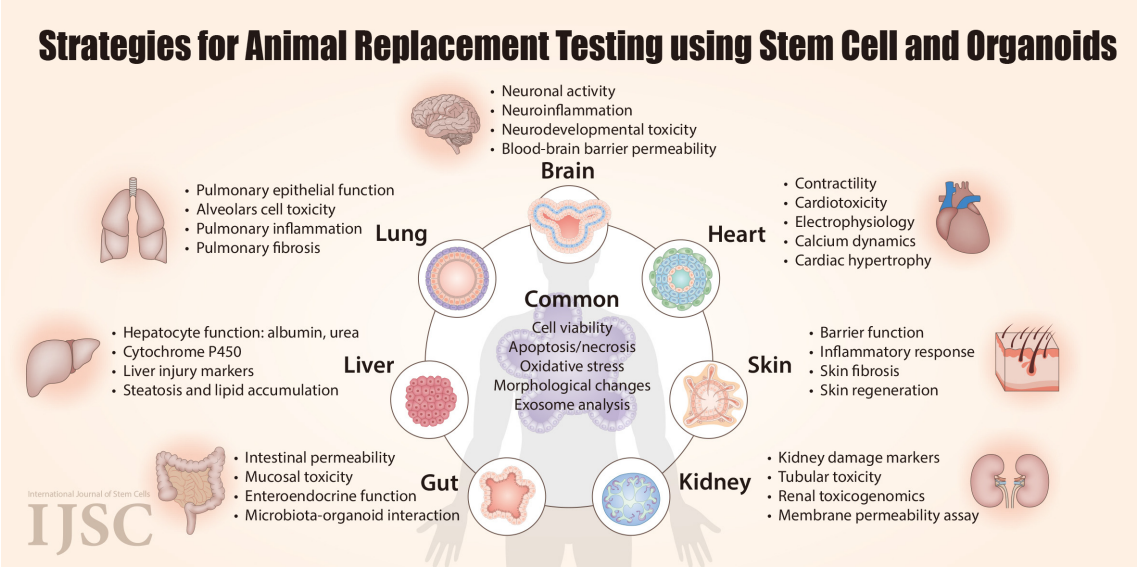

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The increasing ethical concerns and regulatory restrictions surrounding animal testing have accelerated the development of advanced *in vitro* models that more accurately replicate human physiology. Among these, stem cell-based systems and organoids have emerged as revolutionary tools, providing ethical, scalable, and physiologically relevant alternatives. This review explores the key trends and driving factors behind the adoption of these models, such as technological advancements, the principles of the 3Rs (Replacement, Reduction, and Refinement), and growing regulatory support from agencies like the OECD and FDA. It also delves into the development and application of various model systems, including 3D reconstructed tissues, induced pluripotent stem cell-derived cells, and microphysiological systems, highlighting their potential to replace animal models in toxicity evaluation, disease modeling, and drug development. A critical aspect of implementing these models is ensuring robust quality control protocols to enhance reproducibility and standardization, which is necessary for gaining regulatory acceptance. Additionally, we discuss advanced strategies for assessing toxicity and efficacy, focusing on organ-specific evaluation methods and applications in diverse fields such as pharmaceuticals, cosmetics, and food safety. Despite existing challenges related to scalability, standardization, and regulatory alignment, these innovative models represent a transformative step towards reducing animal use and improving the relevance and reliability of preclinical testing outcomes.

Keywords: Animal testing alternatives, Stem cells, Organoids, Toxicity tests

Introduction

The use of animal models in scientific research and drug testing has long been the gold standard for assessing safety, efficacy, and toxicity (1). However, growing ethical concerns, alongside the increasing realization that animal models often fail to accurately predict human responses, have driven the demand for alternative testing methods (2,

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3). According to studies, more than 90% of drugs that succeed in animal trials fail during human clinical testing due to interspecies differences in physiology and drug metabolism (4). In response to these limitations, researchers are turning to advanced *in vitro* methods, particularly those leveraging stem cells and organoids, as promising alternatives to animal testing.

Stem cells, with their ability to differentiate into various specialized cell types, offer an adaptable platform for creating human-specific models (5, 6). Organoids, three-dimensional, self-organizing structures derived from stem cells, closely mimic the architecture and function of human organs. These systems provide a more physiologically relevant environment for studying human biology, disease mechanisms, and drug responses, bridging the gap between traditional 2D cell cultures and animal models (7, 8). To further ensure the reliability and applicability of these models, rigorous model quality control (QC) is crucial (9). This includes the standardization of protocols for differentiation, the evaluation of organoid architecture and functionality, and the application of advanced techniques such as single-cell RNA sequencing and high-content imaging (10, 11). These QC measures help reduce batch-to-batch variability and enhance the reproducibility of experimental results.

Furthermore, the establishment of validated endpoints and indicators for alternative testing models is a key focus of current research and regulatory discussions (12, 13). In animal replacement testing, endpoints such as cellular viability, tissue morphology, and gene expression patterns are critical metrics for assessing model performance and safety outcomes (14). These indicators need to be aligned with regulatory requirements, such as those set forth in the Organisation for Economic Co-operation and Development (OECD) Test Guidelines, to ensure that the results generated from *in vitro* models are directly translatable to human contexts (15). The OECD guidelines offer a structured framework for evaluating chemical and pharmaceutical safety, and the adoption of these guidelines for stem cell and organoid-based models will further facilitate their acceptance and integration into regulatory pipelines (16).

This review explores the current advancements in stem cell and organoid technologies, focusing on their potential to replace animal models in toxicology, pharmacology, and disease research. It will also discuss the integration of these models with cutting-edge technologies such as organ-on-a-chip platforms to enhance their utility and precision. While challenges such as scalability, reproducibility, and regulatory acceptance persist, stem cell-derived organoids, together with standardized QC protocols and well-defined endpoints, represent a significant step toward more ethical and accurate approaches to biomedical research.

Trends and Factors in Animal Replacement Testing Models

The development of animal replacement testing models is driven by ethical, scientific, technological, and regulatory factors. Ethical concerns surrounding animal testing, alongside societal pressure and regulatory guidelines, have spurred the adoption of alternatives. The 3Rs (Replacement, Reduction, and Refinement) principle has become central to this shift, encouraging the use of non-animal methods to minimize ethical dilemmas (17). Traditional animal models often fail to predict human responses due to species differences, leading to high drug failure rates in clinical trials (2, 18). This shortcoming has heightened the need for models that better mimic human physiology and disease.

Technological advancements have been pivotal in this transition. Innovations such as 3D bioprinting, organoids, organ-on-a-chip systems, and CRISPR gene-editing have enabled the creation of more accurate, physiologically relevant models (19, 20). These platforms simulate human biology more effectively, offering improved predictive value for drug efficacy, toxicity, and advancing personalized medicine by allowing for tailored therapeutic approaches based on individual patient profiles (21). Additionally, model standardization research enables the effective operation of biobanks by providing access to standardized, well-characterized collections of human tissues, cells, and genetic material. This standardization ensures consistency and reproducibility across studies, enhancing the reliability of preclinical models and fostering better comparisons in disease modeling and therapeutic testing (Fig. 1).

Regulatory bodies worldwide are spearheading the transition to non-animal testing by establishing comprehensive frameworks and fostering global collaboration to ensure the reliability, reproducibility, and regulatory acceptance of alternative methods. The OECD has developed internationally recognized test guidelines, such as those for *in vitro* skin and eye irritation testing, which serve as benchmarks for evaluating the safety and efficacy of chemicals and pharmaceuticals. These guidelines not only validate non-animal models but also promote their harmonized adoption across diverse regulatory jurisdictions (22). The U.S. Food and Drug Administration (FDA), through its Predictive Toxicology Roadmap, actively supports the integration of cutting-edge technologies, including organoids and *in silico* approaches, into regulatory processes, working closely with industry and academic partners to assess their robustness and translatability (23). Similarly, the

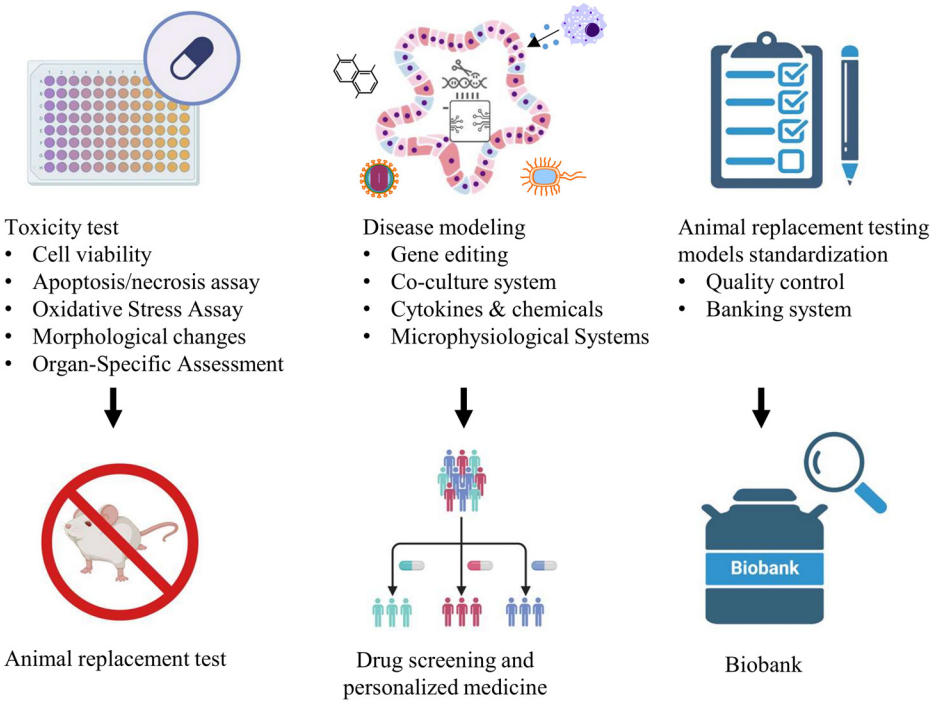

Fig. 1. Cutting-edge approaches in animal replacement test: organoid and stem cell-based testing models. This figure showcases the progression of animal alternative testing models, which advance through key stages such as toxicity evaluation, disease modeling, and standardization studies. These models facilitate the development of alternative testing methods, drug screening, and precision medicine strategies. Additionally, they contribute to the establishment of biobanks, providing access to standardized, well-characterized biological samples. Stem cell-derived organoids and microphysiological systems play a central role in these advancements, helping to replace traditional animal models while improving the accuracy and relevance of preclinical research.

European Medicines Agency aligns its strategies with the European Union's REACH regulation, emphasizing the reduction of animal testing in pharmaceuticals and cosmetics while maintaining stringent safety and efficacy standards (24). The World Health Organization plays a critical role in advancing non-animal methodologies for vaccine and biologics development, standardizing protocols to align with international safety benchmarks and fostering capacity-building programs in low- and middle-income countries. Collectively, these organizations drive innovation, harmonize regulatory standards, and expand the global relevance of stem cell- and organoid-based models, positioning them as pivotal tools in advancing ethical, human-relevant, and scientifically robust testing frameworks.

Industry adoption of non-animal technologies is also on the rise due to their potential to streamline drug development. Advanced *in vitro* models, such as organoids and microphysiological systems (MPS), offer faster and more cost-effective insights into drug safety. Personalized medicine approaches using patient-derived organoids allow for individualized drug testing, particularly in oncology. Overall, the shift toward more ethical and human-relevant models promises to enhance preclinical research by reducing reliance on animal testing while improving outcomes for human health and safety.

Advanced Animal Replacement Testing Models

3D reconstructed tissue models from primary cells with stem cell ability

3D reconstructed tissue models derived from primary cells with stem cell ability represent a significant breakthrough in the quest for reliable alternatives to animal testing. These models mimic the structure and function of human tissues, offering a more relevant platform for toxicity and efficacy testing. 3D reconstructed tissue models have gained recognition and are increasingly being integrated into regulatory frameworks by organizations such as the OECD and FDA. The OECD, for instance, has included several *in vitro* models based on these 3D systems in its Test Guidelines for assessing skin irritation, corrosion, and phototoxicity (Table 1) (25-28). These models provide more human-relevant data, allowing for improved prediction of human biological responses compared to tra-

Table 1. Status of inclusion of reconstructed human skin models in OECD guidelines

	OECD TG 431 (*in vitro* skin corrosion)	OECD TG 439 (*in vitro* skin irritation)	OECD TG 492 (*in vitro* eye irritation using RhCE)
Title	*In vitro* skin corrosion: reconstructed human epidermis test method	*In vitro* skin irritation: reconstructed human epidermis test method	Reconstructed human cornea-like epithelium test method for eye irritation
Initial development date	2004	2010	2019
Type of test	*In vitro* using RhE	*In vitro* using RhE	*In vitro* using RhCE
Objective	Identify corrosive substances and non-corrosive substances	Identify skin irritants (UN GHS Category 2) and non-irritants	Identify chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category)
Exposure time	3 minutes to 4 hours (varies by model)	15 to 60 minutes (varies by model)	10 to 60 minutes (varies by model)
End points	Cell viability measured by MTT assay (cell viability ≤50% for corrosives)	Cell viability measured by MTT assay (cell viability ≤50% for irritants)	Cell viability measured by MTT or WST assays depending on the model
Test models	EpiSkin™, EpiDerm™, SkinEthic™, epiCS®, LabCyte EPI-MODEL24	EpiSkin™, EpiDerm™, SkinEthic™, epiCS®, LabCyte EPI-MODEL24	EpiOcular™, SkinEthic™ HCE, LabCyte CORNEA-MODEL24, MCTT HCE™
Limitations	Cannot fully differentiate between Sub-category 1B and 1C	Does not classify UN GHS Category 3 (mild irritants)	Cannot differentiate between UN GHS Category 1 and 2 chemicals

OECD: Organisation for Economic Co-operation and Development, TG: Test Guidelines, RhCE: reconstructed human cornea-like epithelium, RhE: reconstructed human epidermis, UN GHS: United Nations Globally Harmonized System of Classification and Labelling of Chemicals.

ditional animal tests. Similarly, the FDA has begun accepting data from 3D tissue models in the evaluation of drug toxicity and efficacy (29). These models are used to simulate human tissues in preclinical trials, enhancing the accuracy of safety assessments. The adoption of such models marks a significant step toward reducing animal testing while maintaining or even improving regulatory standards for product safety and effectiveness.

Reconstructed human skin models: Reconstructed human skin models replicate the complex structure and function of human skin, making them essential for studying skin biology, drug absorption, and toxicity (25, 30-32). These models are developed using human keratinocytes and fibroblasts, cultured in a three-dimensional structure that mimics both the epidermal and dermal layers (33). Key features include their physiological relevance, closely resembling native skin in terms of barrier function, tissue architecture, and cellular differentiation, allowing them to accurately mimic natural skin responses to external stimuli such as chemicals, pathogens, and ultraviolet radiation (34). In regulatory toxicology, these models are used following OECD Test Guidelines 439 and 431. TG 439 assesses reversible skin irritation, while TG 431 evaluates irreversible skin corrosion, both using the MTT assay to measure tissue viability (26, 27). Beyond toxicity testing, reconstructed skin models are vital for studying skin diseases like psoriasis, atopic dermatitis, and skin cancer (35-37). They enable detailed investigations into wound healing processes and regenerative medicine by replicating natural skin repair mechanisms (38). Their ability to replicate human-like responses makes them an indispensable tool for advancing dermatological research and therapeutic innovation.

Reconstructed human cornea models: Reconstructed human cornea models are designed to replicate the structure and function of the human cornea, making them essential for studying ocular biology and safety. These models are typically derived from human corneal epithelial cells and closely mimic the barrier function, and layered architecture of native corneal tissue (39). One of their key features is the ability to replicate the corneal epithelium's protective role against environmental insults, pathogens, and chemicals (40, 41). According to OECD Test Guideline 492B, reconstructed human cornea-like epithelium models are standardized for evaluating eye irritation and corrosion potential (42). These models enable researchers to apply test substances and analyze tissue responses, such as cell viability and structural damage, providing human-relevant data without the need for animal testing. In addition to toxicity testing, they are also used to study corneal wound healing, infection, and drug permeability (43, 44). Their ability to simulate key physiological characteristics of the human cornea makes them a valuable tool in ocular

research, facilitating the development of safer pharmaceutical, cosmetic, and chemical products.

Induced pluripotent stem cell-derived cells

Induced pluripotent stem cells (iPSCs), reprogrammed somatic cells capable of differentiating into diverse cell types, have transformed disease modeling and drug development since their discovery by Takahashi et al. (45) in 2007. A major advantage of iPSC-derived cells is their ability to generate patient-specific models, allowing researchers to study disease mechanisms and drug responses tailored to individual genetic backgrounds (46). Their versatility extends to modeling a wide range of diseases, from neurodegenerative and cardiovascular conditions to metabolic disorders, oncology, and immune-related diseases, as well as applications in drug safety and toxicity testing (6, 47-50). However, challenges like genetic and epigenetic changes during reprogramming and the complexity of differentiation protocols can impact the functionality of derived cells (51). Despite these hurdles, iPSCs remain a cornerstone for advancing biomedical research and therapeutic development.

Applications in disease modeling: iPSC-derived cells have deepened our understanding of human diseases by replicating key disease mechanisms. For example, iPSC-derived neurons have modeled Alzheimer's disease and autism spectrum disorders, shedding light on synaptic dysfunctions and aiding drug discovery (47, 52, 53). Similarly, iPSC-derived cardiomyocytes have been used to study long QT syndrome, offering insights into arrhythmias and enabling the testing of targeted therapies (48, 54). In metabolic diseases, pancreatic beta cells and adipocytes derived from iPSCs have advanced research on diabetes and obesity, respectively, revealing critical insights into these disorders (55-58). In oncology, iPSC-derived models have proven instrumental in studying cancers with specific genetic mutations. RET-rearranged non-small cell lung cancer models have shown sensitivity to inhibitors like selpercatinib (59), while PTEN-deficient glioblastoma models have facilitated the development of PI3K/AKT/mTOR inhibitors (60). Similarly, iPSC-derived megakaryocytes with JAK2 mutations have supported the evaluation of JAK-STAT inhibitors in myeloproliferative neoplasms (61). These models are invaluable for mimicking disease-specific phenotypes and driving therapeutic innovations.

Applications in toxicity testing: iPSC-derived models offer predictive, human-relevant platforms for toxicity testing, reducing reliance on animal studies. Cardiomyocytes derived from iPSCs are extensively used to assess drug-induced cardiotoxicity, such as the effects of doxorubicin and tyrosine kinase inhibitors (62). iPSC-derived hepatocytes replicate liver-specific metabolic pathways, crucial for predicting drug-induced liver injury caused by compounds like acetaminophen (63, 64). Co-culture systems integrating Kupffer cells further enhance physiological relevance by capturing immune-mediated toxicity (65). In neurotoxicity studies, iPSC-derived neural cells are used to evaluate the effects of drugs and environmental toxins on the central nervous system, revealing mechanisms like oxidative stress and apoptosis (66, 67). Additionally, iPSC-derived alveolar epithelial cells have advanced pulmonary toxicity research by modeling the alveolar-capillary barrier and aiding the study of inhaled drugs and environmental toxins (68). These systems significantly improve the accuracy of toxicity predictions, contributing to safer drug development.

Organoids

Organoids are three-dimensional structures derived from stem cells that replicate the architecture and functionality of real organs (7). These innovative systems have become invaluable tools for studying organ development, disease mechanisms, and drug responses. Depending on their origin, organoids can be generated from human pluripotent stem cells (hPSCs) or adult stem cells (69).

Organoids derived from hPSCs, including embryonic stem cells and iPSCs, are highly versatile due to their ability to differentiate into a wide range of cell types (70). This makes them particularly useful for recapitulating early developmental stages of organs and studying congenital diseases (71). For instance, liver organoids generated from hPSCs have been employed to model metabolic dysfunction-associated steatohepatitis, shedding light on the cellular mechanisms underlying this chronic liver disease (72). Similarly, brain organoids derived from hPSCs have been used to investigate the impact of genetic mutations on cortical development, such as RAB39b-PI3K-mTOR pathway dysregulation, which has been linked to autism spectrum disorder and macrocephaly (73).

In contrast, organoids generated from adult stem cells more closely mimic mature organ physiology but are typically restricted to specific tissue types (74). A significant advancement in this field is the development of patient-derived organoids (PDOs), which are generated from adult stem cells or tumor cells obtained directly from patient tissues (75). PDOs retain the genetic and phenotypic characteristics of the donor, enabling researchers to study diseases in a patient-specific context and develop personalized therapeutic strategies (76). For example, PDOs derived from lung tissue have been used to model SARS-CoV-2 infection, providing insights into viral interactions with lung-resident immune cells, while intestinal PDOs

have facilitated studies on Crohn's disease by revealing how T cell-driven epithelial cell death contributes to its pathogenesis (77-79). Furthermore, PDOs have proven invaluable in cancer research, with human lung adenocarcinoma-derived organoids serving as a platform for drug screening and pancreatic cancer organoids recapitulating disease features to allow personalized drug testing (80, 81). These models not only deepen our understanding of tumor biology and disease mechanisms but also pave the way for precision medicine by tailoring treatments to individual patients.

Organoid systems have also proven highly valuable in toxicity testing due to their tissue-like organization and functionality. hPSC-derived kidney organoids, for example, have been used to model acute nephrotoxic injury caused by cisplatin, capturing the complex interactions between renal cell types (82). Brain organoids generated from hPSCs have been utilized to study developmental neurotoxicity, allowing researchers to screen environmental neurotoxicants and their effects on neurodevelopmental disorders such as (83). PDOs further extend these applications, as they can be used to assess the toxicity of drugs on patient-specific tissues, providing a personalized approach to evaluating treatment safety and efficacy. For example, oral mucosa PDOs have modeled methotrexate-induced toxicity in pediatric leukemia, while retinoblastoma PDOs have demonstrated sunitinib's efficacy with minimal toxicity (84, 85). These cases highlight the potential of PDOs in precision medicine for optimizing treatment strategies.

While hPSC-derived organoids excel in developmental studies and generalized disease modeling, PDOs are particularly valuable for precision medicine. Together, these systems complement each other, offering a comprehensive platform to advance our understanding of human biology, disease mechanisms, and therapeutic strategies. Despite some challenges, such as variability in culture conditions and incomplete representation of systemic interactions, organoid technology continues to evolve, bridging the gap between *in vitro* studies and clinical applications.

MPS: dynamic platforms built on stem cell and organoids

MPS, often referred to as "organ-on-a-chip" technology, are advanced platforms that integrate microfluidic channels to emulate organ-level functions and physiological processes such as blood flow, nutrient exchange, and mechanical forces. These systems provide a dynamic and controlled environment that surpasses traditional cell cultures by closely mimicking *in vivo* conditions (86). MPS can incorporate human cells derived from iPSCs, organoids, or patient-specific tissues, enabling precise modeling of individual organ functions and inter-organ interactions (87).

Compared to organoids, MPS offer distinct advantages. They provide enhanced control over fluid dynamics and mechanical forces, supporting more reproducible experimental outcomes. Furthermore, MPS facilitate the study of systemic interactions between organs, such as liver-heart or gut-brain communication, offering deeper insights into processes like drug metabolism, toxicity, and disease mechanisms (88). Their scalability and suitability for high-throughput screening also make them indispensable tools in drug development and personalized medicine. By bridging the gap between organoids and traditional *in vitro* models, MPS represent a crucial step forward in replicating the complexity of human physiology for both research and therapeutic applications (89).

For example, MPS for the heart, liver, and brain provide advanced models to study organ-specific functions and drug responses. Heart MPS assess cardiac contractility and electrical conduction using techniques like calcium imaging, allowing for the evaluation of cardiotoxicity and heart disease mechanisms (90). Liver MPS utilize hepatocyte viability and enzyme activity, particularly cytochrome P450, to investigate drug metabolism and hepatotoxicity, offering insights into toxic metabolite formation (91). Brain MPS focus on neuronal firing rates and synaptic activity to assess neurotoxicity, replicating key aspects of brain function and enabling research into neurodegenerative diseases and drug effects on neural networks (92, 93).

There is a growing trend in MPS research towards developing multi-organ systems, where multiple organ models are integrated on a single chip to study inter-organ communication (94). These multi-organ platforms, often referred to as "body-on-a-chip" can simulate complex systemic processes like drug absorption, distribution, metabolism, and excretion (95, 96). These integrated systems provide a more holistic view of human physiology and are particularly useful for assessing the overall toxicity and therapeutic potential of drugs.

QC in animal replacement testing models

QC in animal replacement testing models, such as cells, organoids and MPS, is essential to ensure reproducibility, reliability, and regulatory compliance. Standardizing protocols for cell culture, differentiation, and maintenance is crucial for consistency across batches. QC metrics include the quality of cells, where genetic stability, purity, and absence of contamination (e.g., mycoplasma) must be verified (Fig. 2) (97). Functional validation tests, such as enzyme activity in liver models or contractility in heart models or barrier function in skin models, ensure that the models

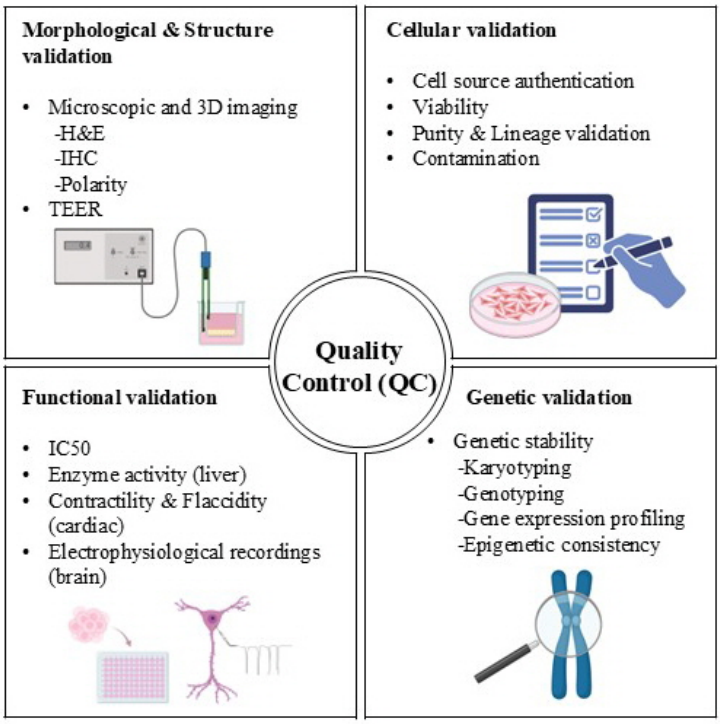

Fig. 2. Comprehensive quality control (QC) strategies for animal replacement testing models. This figure illustrates the essential QC strategies required to ensure the reliability and reproducibility of animal replacement testing models. It outlines various QC stages, including morphological and structural validation, as well as genetic and functional assessments of stem cell-derived organoids. Additionally, the diagram emphasizes cellular validation and batch-to-batch consistency checks. Key evaluations include tissue architecture analysis and specific organ functionality tests, such as enzyme activity for liver organoids or barrier integrity for skin models. These QC measures are critical for regulatory approval, ensuring that *in vitro* models meet the required standards for toxicity testing, disease modeling, and drug screening. IHC: immunohistochemistry, TEER: trans-epithelial electrical resistance.

accurately mimic human physiology (98). Morphological assessments, like tissue organization and 3D structure integrity, are routinely checked using imaging techniques (9). Reproducibility is assessed through batch-to-batch comparisons in gene expression and functional outcomes, with acceptable variation thresholds predefined. Alignment with regulatory standards like good manufacturing practice or glucagon-like peptide (GLP) is critical for preclinical use (99). Ethical sourcing and traceability of human cells, along with robust data management systems, further support QC efforts, ensuring transparency and accountability throughout the model development process.

Strategic challenges and future directions

Despite their transformative potential, stem cell-derived models and organoids face several scientific and practical challenges that hinder their full replacement of animal models in evaluation studies. One major limitation is their inability to replicate the complexity of *in vivo* conditions, including vascularization and microenvironmental interactions, which are essential for long-term viability and accurate modeling of organ functions (100). Current efforts, such as integrating endothelial cells for vascularization or using microfluidic systems to simulate nutrient and oxygen delivery, show promise but lack consistent and reproducible results (101). Additionally, these models struggle to represent systemic interactions, such as the gut-liver or brain-heart axis, which are critical for studying complex diseases and drug effects. Multi-organ-on-a-chip technologies and co-culture systems aim to address this but face challenges related to scalability, standardization, and monitoring (94). Genetic and epigenetic drift during long-term culture further compromises reproducibility, while batch-to-batch variability caused by donor heterogeneity and technical inconsistencies complicates their consistent application (102). These limitations are exacerbated when modeling chronic and long-term conditions, such as fibrosis or neurodegeneration, as prolonged cultures often result in cellular senescence and loss of tissue functionality (103). Ethical concerns about human tissue sourcing and logistical barriers, including high costs and technical expertise, also hinder accessibility, particularly in resource-limited settings. Regulatory acceptance re-

mains a significant challenge, with the lack of harmonized standards, validated endpoints, and large-scale comparative studies delaying their routine use in drug development and toxicity testing (97). Addressing these multifaceted challenges requires interdisciplinary collaboration and technological innovation to enhance scalability, reproducibility, and regulatory alignment. By overcoming these barriers, stem cell- and organoid-based models can fulfill their potential as ethical, human-relevant, and scientifically robust alternatives to animal models, transforming biomedical research and preclinical testing.

Advanced Animal Replacement Testing Methods

Toxicity assessment strategies

Toxicity assessment is a critical component of drug development and safety evaluation. Traditional animal testing often fails to accurately predict human responses, leading to high failure rates in clinical trials (104). The use of advanced animal replacement models, such as organoids and MPS, enhances the reliability of toxicity predictions by providing human-relevant data and reducing reliance on animal studies.

When selecting toxicity assessment indicators, several factors should be considered to ensure effectiveness. Firstly, the relevance to human biology is crucial; indicators must reflect human physiological and biochemical processes to ensure that results are translatable to human health outcomes (105). Secondly, the sensitivity and specificity of the indicators are important, as they should be able to detect low levels of toxicity while minimizing false positives or negatives. Additionally, choosing indicators that provide mechanistic insights into the underlying mechanisms of toxicity is essential for understanding how a compound may affect cellular functions or lead to adverse effects.

Statistical robustness is another key consideration, as the metrics selected should have a strong statistical foundation to ensure reliable and reproducible data. Furthermore, alignment with regulatory acceptance is necessary, facilitating the acceptance of these indicators in safety evaluations and ensuring compliance with relevant regulations (106). Lastly, practical aspects such as feasibility and cost-effectiveness should also be taken into account, including the availability of resources and the overall cost of measurement. By incorporating these factors into the selection of toxicity assessment indicators, researchers can enhance the predictive power of their models and improve safety evaluations in drug development.

Common toxicological assessment strategies: Several general toxicity assays are widely used across different organ systems to assess overall cellular responses to toxic exposures. Cell viability assays such as adenosine triphosphate (ATP) production, MTT reduction, and lactate dehydrogenase (LDH) release are commonly used to measure changes in cell health and viability (Table 2) (107). These assays detect reduced ATP and MTT levels or increased LDH release, which are indicative of cell damage or death (108). For more mechanistic insights, apoptosis and necrosis assays are employed using markers like caspase activation or Annexin V/PI staining to differentiate between programmed cell death and necrotic injury (109, 110). Furthermore, oxidative stress markers such as glutathione depletion and increased reactive oxygen species production can identify oxidative damage resulting from toxicants (111, 112).

Additionally, structural and functional changes in cells can be evaluated using morphological assessments like H&E staining and immunofluorescence staining, while exosome analysis investigates the intercellular signaling pathways affected by toxic substances (113, 114). Exosomes, carrying specific proteins and microRNAs, have been shown to play a role in systemic toxicity responses, making them promising markers for toxicity assessment (115, 116).

Organ-specific toxicity assessment strategies and prior research:

a. Liver

The liver, being the primary organ for detoxification and drug metabolism, is highly susceptible to a wide range of toxic substances. To evaluate hepatotoxicity, liver-specific assays focus on both functional and damage markers (117). Hepatocyte function assays measure the production of liver-specific secretions such as albumin and urea, while Cytochrome P450 enzyme activity assays assess the metabolic capacity of hepatocytes through specific enzyme activities like CYP3A4 and CYP1A2 (118, 119). Liver injury markers such as alanine aminotransferase and aspartate aminotransferase are frequently used to detect hepatocellular injury, as elevated levels are indicative of liver cell damage (120).

Moreover, hepatotoxicity can be further characterized by evaluating bile acid transport using Bile Salt Export Pump activity assays or lipid accumulation and steatosis through Oil Red O staining (121-123). In cases of chronic liver injury, liver fibrosis assays using markers like collagen deposition and α-smooth muscle actin (α-SMA) expression can identify the extent of fibrogenesis, making these markers crucial for evaluating long-term hepatic toxicity (124).

b. Kidney

Kidney toxicity assessment focuses on evaluating renal

function and detecting early signs of nephrotoxicity. Kidney function assays, such as albumin and glucose reabsorption tests, provide information on tubular function, where a decrease in these parameters suggests tubular damage (125, 126). Biomarkers like kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin are highly sensi-

Table 2. Toxicity assessment indicators by organ

	Toxicity assessment method	Key evaluation indicators	Description	References
Common	Cell viability assay	ATP production MTT assay LDH release	Evaluates changes in cell viability due to toxic substances. Decreased ATP and MTT levels, and increased LDH release indicate cell damage	(30, 107, 108)
	Apoptosis/necrosis assay	Caspase activation Annexin V/PI staining TUNEL assay	Detects cell death mechanisms, such as apoptosis (caspase activation) and necrosis (membrane permeability changes)	(109, 110)
	Oxidative stress assay	GSH levels ROS production	Assesses oxidative stress responses. Decreased GSH and increased ROS can be induced by toxic reactions, allowing the evaluation of oxidative damage in various organ cells	(111, 112)
	Morphological changes	H&E staining Immunofluorescence staining Cell polarity assessment	Evaluates structural and histological changes in cells due to toxic substances. Uses H&E staining to assess cell structure and immunofluorescence staining to confirm specific protein expression	(113, 114)
	Exosome analysis	Exosome marker expression (e.g., CD63, CD81) microRNA analysis	Evaluates intercellular signaling and toxicity response pathways. Analyzes toxicity response through changes in specific markers or microRNAs contained in exosomes	(115, 116)
Liver	Hepatocyte function assay	Albumin secretion Urea production	Assesses liver function by measuring hepatocyte-specific secretions such as albumin and urea	(117)
	Cytochrome P450 enzyme activity assay	CYP3A4 activity CYP1A2 activity	Evaluates the drug-metabolizing capacity of liver organoids by measuring specific cytochrome P450 enzyme activities	(118, 119)
	Liver injury markers assay	ALT AST	Measures liver cell damage markers (ALT, AST) released during hepatocyte injury	(120)
	Bile acid transport assay	BSEP activity	Assesses the functionality of bile acid transport mechanisms, an important aspect of hepatotoxicity	(121)
	Steatosis and lipid accumulation assay	Oil Red O staining Triglyceride content	Evaluates lipid accumulation and steatosis, often induced by toxicants, using lipid-specific staining and content measurements	(122)
	Cholestasis assay	Bile canaliculi formation Bile acid secretion	Assesses bile canaliculi structure and function, key to identifying cholestatic effects of drugs and toxicants	(123)
	Liver fibrosis assay	Collagen deposition (Sirius Red staining) α-SMA expression	Evaluates fibrogenesis by detecting collagen deposition and fibrotic markers, indicative of chronic liver injury	(124)
Kidney	Kidney function assay	Albumin reabsorption Glucose reabsorption	Assesses tubular cell function by measuring protein and glucose reabsorption, with reductions indicating kidney injury	(125, 126)
	Kidney damage markers	KIM-1 NGAL	Evaluates kidney damage using specific biomarkers, where increased KIM-1 and NGAL levels indicate renal injury	(127)
	Tubular toxicity assay	γ-GT activity	Measures tubular toxicity, with increased γ-GT activity indicating tubular damage	(128, 131)
	Membrane permeability assay	TEER changes	Assesses membrane permeability of tubular cells, with decreased TEER indicating membrane damage or dysfunction	(129, 130)
	Renal toxicogenomics	Kidney-specific genes (e.g., *AQP1, SLC22A2*) Toxicity response genes (e.g., *NFE2L2, HMOX1*)	Analyzes gene expression changes specific to renal function and toxicity responses	(82, 132, 133)

Table 2. Continued

	Toxicity assessment method	Key evaluation indicators	Description	References
Brain	Neuronal activity assay	Calcium influx changes Spontaneous electrical activity monitoring	Evaluates functional neuronal activity through calcium signaling and electrical activity, indicative of neurotoxicity	(134)
	Neuroinflammation assay	IL-6, TNF-α secretion	Assesses neuroinflammatory responses via cytokine secretion from neural and glial cells	(135, 136)
	Neurodevelopmental toxicity assay	Neuronal differentiation markers (e.g., TUJ1, MAP2, MAPT) Synapse formation proteins (e.g., Synapsin, PSD-95)	Evaluates neurodevelopmental toxicity by assessing changes in neuronal differentiation and synaptic protein expression	(137-139)
	Blood-brain barrier permeability assay	TEER measurement Permeability changes (e.g., FITC-dextran assay)	Assesses blood-brain barrier integrity and permeability, with decreased TEER or increased FITC-dextran passage indicating barrier disruption	(140)
Gut	Intestinal permeability assay	TEER measurement FITC-dextran leakage test	Assesses intestinal barrier function, with decreased TEER or increased FITC-dextran leakage indicating barrier dysfunction	(141)
	Mucosal toxicity assay	Muc2 (Mucin) secretion Mucosal layer thickness	Evaluates mucosalbarrier function, where reduced Muc2 secretion or mucosal layer thinning indicates mucosal toxicity	(142)
	Enteroendocrine function assay	GLP-1, GIP secretion	Measures the secretory function of enteroendocrine cells, with altered hormone levels indicating functional impairment	(143)
	Microbiota-organoid interaction assay	Specific microbiota changes Microbial metabolites (e.g., short-chain fatty acids)	Assesses interactions between intestinal organoids and microbiota, with changes in microbial composition or metabolites indicating dysbiosis or toxicity	(144)
Heart	Contractility assay	BPM Fractional shortening	Evaluates contraction and relaxation function of cardiac organoids, with reduced contractility indicating cardiotoxic effects	(145)
	Cardiotoxicity assay	cTnI LDH release	Measures cardiomyocyte damage using specific biomarkers, with increased cTnI and LDH levels indicating cardiac injury	(146)
	Electrophysiology assay	APD FPD	Evaluates electrophysiological properties of cardiac organoids, with prolonged APD or FPD indicating proarrhythmic or cardiotoxic effects	(147)
	Calcium dynamics assay	Calcium transient analysis	Assesses calcium influx and efflux patterns in cardiomyocytes, with altered calcium transients indicating cardiotoxicity	(148)
	Cardiac hypertrophy assay	BNP expression Cell size analysis	Evaluates hypertrophic responses, with increased BNP expression and cell size indicating pathological cardiac hypertrophy	(149)
Skin	Barrier function assay	TEER measurement TEWL	Assesses skin barrier function and water loss, with reduced TEER and increased TEWL indicating barrier damage	(150)
	Inflammatory response assay	IL-1β, IL-6, TNF-α secretion	Measures inflammatory responses by cytokine secretion levels, with increased secretion indicating skin inflammation	(151)
	Skin fibrosis assay	Collagen I and III expression α-SMA expression	Evaluates fibrotic responses, with increased collagen and α-SMA expression indicating fibrosis	(152)
	Skin sensitization assay	Th1/Th2 cytokine secretion (e.g., IL-4, IL-12) Dendritic cell activation	Assesses allergic reactions, with altered Th1/Th2 cytokine levels and dendritic cell activation indicating skin sensitization	(153)
	Skin regeneration assay	Wound healing rate Keratinocyte migration & proliferation	Evaluates skin regeneration, with increased wound healing rate and keratinocyte migration indicating enhanced regenerative capacity	(154)

Table 2. Continued

	Toxicity assessment method	Key evaluation indicators	Description	References
Lung	Pulmonary epithelial function assay	MUC5AC, MUC1 expression	Evaluates mucus production, with altered MUC5AC or MUC1 levels indicating functional impairment in pulmonary epithelial cells	(155)
	Alveolar cell toxicity assay	SP-C expression Aquaporin-5 expression	Assesses alveolar cell function, with reduced SP-C or Aquaporin-5 indicating alveolar cell damage	(156, 157)
	Pulmonary inflammation assay	IL-8, IL-6, TNF-α secretion	Measures inflammatory responses in lung models, with increased cytokine levels indicating pulmonary inflammation	(158)
	Pulmonary fibrosis assay	Collagen I and III expression α-SMA expression TGF-β1 expression	Evaluates fibrotic responses, with increased collagen, α-SMA, and TGF-β1 levels indicating pulmonary fibrosis	(159)
	Morphological changes in lung cells	E-cadherin, vimentin expression Cell polarity changes (e.g., ZO-1 staining)	Assesses EMT and morphological changes, with reduced E-cadherin and increased vimentin indicating EMT and polarity loss	(77, 160)

ATP: adenosine triphosphate, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, LDH: lactate dehydrogenase, GSH: glutathione, ROS: reactive oxygen species, CYP: cytochrome p450, ALT: alanine aminotransferase, AST: aspartate aminotransferase, BSEP: bile salt export pump, α-SMA: α-smooth muscle actin, KIM-1: kidney injury molecule-1, NGAL: neutrophil gelatinase-associated lipocalin, γ-GT: γ-glutamyl transferase, TEER: trans-epithelial electrical resistance, *AQP1*: aquaporin-1, *SLC22A2*: solute carrier family 22 member 2, *NFE2L2*: nuclear factor erythroid 2-related factor 2, *HMOX1*: heme oxygenase 1, IL-6: interleukin-6, TNF-α: tumor necrosis factor-α, TUJ1: neuronal class III β-tubulin, MAP2: microtubule-associated protein 2, MAPT: microtubule-associated protein tau, PSD-95: post-synaptic density protein 95, FITC: fluorescein isothiocyanate, GLP-1: glucagon-like peptide-1, GIP: gastric inhibitory polypeptide, BPM: beats per minute, cTnI: cardiac troponin I, APD: action potential duration, FPD: field potential duration, BNP: brain natriuretic peptide, TEWL: transepidermal water loss, IL-1β: interleukin-1β, MUC5AC: mucin 5AC, MUC1: mucin 1, SP-C: surfactant protein C, TGF-β1: transforming growth factor-β1, ZO-1: zonula occludens-1, EMT: epithelial-mesenchymal transition.

tive indicators of acute kidney injury, with increased levels pointing to renal damage (127).

Additionally, tubular toxicity assays measuring γ-glutamyl transferase activity are used to evaluate damage to tubular cells, while membrane permeability assays utilizing trans-epithelial electrical resistance (TEER) measurements detect changes in membrane integrity (128-131). For a deeper understanding of molecular alterations, renal toxicogenomics assays analyze the expression of kidney-specific genes (e.g., *AQP1*, *SLC22A2*) and toxicity response genes such as *NFE2L2* and *HMOX1*, providing insights into the molecular pathways involved in renal toxicity (132, 133).

c. Brain

Neurotoxicity assessment poses unique challenges due to the complexity of the central nervous system. Functional assays such as neuronal activity assays, which measure calcium influx and spontaneous electrical activity, are used to detect disturbances in neuronal signaling (134). Neuroinflammation assays, on the other hand, measure cytokine secretion (e.g., IL-6, TNF-α) to assess the inflammatory responses within neural and glial cells (135, 136). For developmental neurotoxicity, the expression of neuronal differentiation markers (e.g., Tuj1, MAP2) and synaptic proteins (e.g., Synapsin, PSD-95) is evaluated to detect alterations in neuronal development and synapse formation (137-139). Additionally, blood-brain barrier permeability assays such as TEER measurements and FITC-Dextran passage assays are employed to assess the integrity of the blood-brain barrier, with changes indicating potential disruptions that could lead to increased neurotoxicity (140).

d. Gut

Gut-specific toxicity assessment is centered on evaluating intestinal barrier function and mucosal health. Intestinal permeability assays such as TEER measurements and FITC-Dextran leakage tests are used to assess barrier integrity, where a decrease in TEER or an increase in FITC-Dextran passage indicates barrier dysfunction (141). Mucosal toxicity assays measure mucin secretion (e.g., Muc2) and mucosal layer thickness to evaluate the impact of toxicants on mucosal integrity (142).

Enteroendocrine function assays, which measure the secretion of hormones like GLP-1 and gastric inhibitory polypeptide, offer insights into changes in hormone pro-

duction, highlighting potential disruptions in gut function and metabolic regulation (143). Microbiota-organoid interaction assays analyze alterations in microbial composition and metabolites, allowing researchers to evaluate the effects of toxicants on gut microbiota and overall gut health (144).

e. Heart

Cardiotoxicity is assessed using a range of functional and molecular markers that reflect cardiac health and damage. Contractility assays measure beats per minute and fractional shortening to evaluate the contractile function of cardiac cells (145). In addition, cardiac troponin I and LDH release are commonly used biomarkers to detect cardiomyocyte damage, with elevated levels indicating cardiac injury (146).

Electrophysiology assays such as action potential duration and field potential duration are critical for identifying proarrhythmic effects, while calcium dynamics assays evaluate calcium influx and efflux patterns in cardiomyocytes, with abnormalities in calcium transients indicating cardiotoxicity (147, 148). Additionally, elevated levels of brain natriuretic peptide and atrial natriuretic peptide in response to toxic exposure can serve as important biomarkers for cardiac stress and hypertrophy, reflecting underlying cardiotoxic effects (149).

f. Skin

The skin serves as a protective barrier, and its toxicity assessment involves evaluating barrier function and inflammatory responses. Barrier function assays use TEER measurements and transepidermal water loss tests to assess the integrity of the skin barrier, while inflammatory response assays focus on cytokine secretion (e.g., IL-1β, IL-6, TNF-α) to detect skin inflammation (150, 151).

For fibrotic responses, markers such as collagen I and III expression, along with α-SMA expression, are used to identify skin fibrosis (152). Skin sensitization assays measure Th1/Th2 cytokine levels and dendritic cell activation to evaluate allergic reactions, while skin regeneration assays assess wound healing rates and keratinocyte migration, with increased rates indicating enhanced regenerative capacity (153, 154).

g. Lung

Lung-specific toxicity assessment methods focus on evaluating both alveolar and pulmonary epithelial functions. Pulmonary epithelial function assays measure mucus production markers such as MUC5AC and MUC5B, with changes indicating epithelial dysfunction (155). Alveolar cell toxicity assays monitor the expression of surfactant proteins like SP-C and Aquaporin-5, with reduced expression suggesting alveolar cell damage (156, 157).

Pulmonary inflammation assays measure cytokine secretion (e.g., IL-8, IL-6, TNF-α), while pulmonary fibrosis assays detect collagen deposition, α-SMA expression, and TGF-β1 levels to identify fibrotic changes (158, 159). Furthermore, morphological changes in lung cells are evaluated through markers such as E-cadherin and vimentin expression, where reduced E-cadherin and increased vimentin indicate epithelial-mesenchymal transition and loss of cell polarity (77, 160).

In summary, organ-specific toxicity assessment relies on a diverse array of functional assays, molecular markers, and morphological analyses to provide a comprehensive understanding of toxic responses. Incorporating these strategies allows researchers to predict human toxicity more accurately and to develop safer therapeutic and chemical agents based on organ-specific insights.

Efficacy assessment strategies

Advanced animal replacement efficacy assessment is an innovative approach in evaluating cosmetics, food products, and therapeutic agents, focusing on minimizing or eliminating animal testing through the use of cutting-edge methodologies. These advanced models include *in vitro* systems such as organoids, MPS, and computational simulations, which more accurately mimic human biological responses, thus enhancing the reliability and relevance of efficacy evaluations.

Cosmetics: In the cosmetics industry, the enforcement of bans on animal testing has accelerated the adoption of advanced animal replacement testing methods. These innovative efficacy evaluations concentrate on the ability of ingredients to enhance skin health, improve aesthetic qualities, and provide protection against environmental stressors. Techniques such as 3D reconstructed skin models and high-throughput screening of bioactive compounds are commonly used to assess skin hydration, anti-aging effects, hair-loss prevention, and potential irritants, all without relying on animal testing (161). Recent studies have developed hair-bearing skin organoids that more accurately replicate the human hair follicle environment, making them invaluable for cosmetic and drug evaluations (162). These *in vitro* systems offer a more relevant alternative to traditional animal models for testing compounds like minoxidil by enabling the evaluation of key factors such as hair shaft elongation, cell viability, and hair-specific gene expression to assess the effectiveness of hair care products (163).

Food: For food products, efficacy assessment focuses on evaluating the health benefits of various ingredients, such as their antioxidant properties and ability to support gut

health. Advanced methods, including *in vitro* digestion models, cellular assays for nutrient bioavailability, and microbiome analysis, provide insights into how food components affect gut flora. Recently, gut–organ-axis-on-a-chip systems have been utilized in food research to examine interactions among dietary components and organs (164). For example, gut-liver organ-on-a-chip systems have demonstrated their effectiveness in assessing drug pharmacokinetics, modeling first-pass metabolism, and evaluating dietary components' interactions, providing valuable insights into their health benefits and metabolic processes (165, 166).

Therapeutics: Advanced animal replacement models, like human-derived cell lines, organoids, and MPS, allow for more accurate drug efficacy and toxicity assessments by replicating organ functions in a human-relevant context. Cancer organoids, which retain the structure and genetic traits of patient tumors, are increasingly used for high-throughput drug screening, helping to identify tumor-specific vulnerabilities (167). A recent study reported that patient-derived breast cancer organoids from various subtypes (luminal A, luminal B, HER2-enriched, and triple-negative) were used to identify drug-resistant populations, with findings showing that inhibiting YAP activation can help restore chemosensitivity in these resistant organoids (168). Similarly, in colorectal cancer, PDOs have demonstrated that resistance to standard chemotherapy, such as 5-FU and Irinotecan, is mediated by Hedgehog signaling pathways, and the use of Hedgehog inhibitors can effectively reduce drug resistance and cancer stem cell marker expression, highlighting their potential as a combinational therapy in overcoming treatment resistance (169).

Conclusion

Stem cell-based models and organoids have established a solid foundation for replacing traditional animal testing by closely mimicking human physiological and pathological conditions. These advanced models, including iPSC-derived cells, three-dimensional tissue constructs, and organ-on-a-chip platforms, offer new opportunities for evaluating drug efficacy, toxicity, and disease mechanisms in an ethical and scientifically relevant manner. However, challenges such as scalability, standardization, and regulatory acceptance still need to be addressed to enable broader adoption. Overcoming these hurdles through the development of robust QC standards and alignment with existing regulatory frameworks is essential for accelerating the transition to animal-free testing paradigms. Ultimately, the shift from animal models to advanced *in vitro* systems signifies a transformative step in preclinical research, with the potential to improve the reliability and relevance of experimental outcomes while reducing the ethical burden associated with animal testing. Continued efforts to optimize these models and expand their applications will pave the way for a new era in biomedical research that prioritizes human-relevant testing strategies and adheres to the principles of the 3Rs.

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Potential Conflict of Interest

There is no potential conflict of interest to declare.

Authors' Contribution

Conceptualization: CJL. Funding acquisition: YAR, JHJ. Writing – original draft: CJL, YN. Writing – review and editing: YN, YAR, JHJ.

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