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Review Article

# Patient-derived induced pluripotent stem cell organoids for amyotrophic lateral sclerosis drug discovery

**Wenyan Li[a,b,1], Jinqi Liu[c,1], Wenting Li[d], Lina Sun[c], Hao Zhang[e,*], Lei Gao[f,*] and Chong Gao[a,*]**

[a]Institute of Brain and Cognitive Science, School of Medicine, Hangzhou City University, Hangzhou, China
[b]College of Integrated Chinese and Western Medicine, Hebei University of Chinese Medicine, Shijiazhuang, China
[c]State Key Laboratory of Cognitive Neuroscience and Learning, Beijing Normal University, Beijing, China
[d]Department of Pharmacy, The Eighth Affiliated Hospital, Sun Yat-Sen University, Shenzhen, China
[e]Department of Neurology, Motor Neuron Disease Diagnosis and Treatment Center, Affiliated Hangzhou First People's Hospital, School of Medicine, Westlake University, Hangzhou, China
[f]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China

[1]Co-first author with equal contribution.
***Correspondence:** gaoc@hzcu.edu.cn (C. Gao); raygaolei@smu.edu.cn (L. Gao); zhanghao@hospital.westlake.edu.cn (H. Zhang)

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## ABSTRACT

Complex biological mechanisms and unidentified therapeutic targets for amyotrophic lateral sclerosis (ALS) significantly hinder the development of effective treatments. Given these challenges, reliable disease models that accurately replicate ALS phenotypes with relevant biological underpinnings are essential for advancing precision medicine in ALS. Patient-derived induced pluripotent stem cell (iPSC) organoids have emerged as an innovative tool for disease modeling and drug evaluation. Growing evidence highlights the advantages of organoids in replicating ALS phenotypes and supporting drug development. However, challenges remain in utilizing organoids for ALS drug testing and other neurodegenerative diseases. In this review we summarize the current progress in ALS model development, encompassing both *in vitro* and *in vivo* non-human models, as well as iPSC-derived human models. Furthermore, within the context of ALS drug screening, we discuss critical considerations for applying organoids to evaluate disease-associated phenotypes and to accurately reflect disease-related symptoms.

**Keywords:** Amyotrophic lateral sclerosis, Disease models, Organoids, High-throughput screening

## 1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a lethal, progressive neurodegenerative disease characterized by the loss of upper and lower motor neurons [1]. The pathologic hallmarks of ALS include motor neuron atrophy, gliosis, and the accumulation of misfolded proteins, such as TAR DNA-binding protein 43 (TDP-43) aggregates, dipeptide repeat proteins, and fused in sarcoma (FUS) protein mislocation [2]. The loss of motor neural function results in muscle weakness, atrophy, and eventual paralysis, with respiratory failure being the primary cause of mortality. Current medications only provide limited effects to prevent progressive neurodegeneration.

The incidence of amyotrophic lateral sclerosis (ALS) ranges from 0.6–3.8 per 100,000 individuals annually worldwide. Greater than 30 at-risk genes have been identified concerning the pathogenesis of ALS [3-6], which accounts for the complex mechanisms leading to ALS [7]. The heterogeneity of ALS phenotypes and difficulty in obtaining biopsy samples from the central nervous system (CNS) pose challenges in advancing the understanding of ALS and the development of curable treatment. Current strategies for drug screening by the pharmaceutical industry are mainly based on identified ALS targets, the list of which is incomplete, especially sporadic ALS cases. An alternative strategy for drug screening based on phenotypic analysis requires a reliable disease model associated with disease symptoms.

Non-human models, including yeast, flies, worms, zebrafish, and rodents, have offered insights into the cellular phenotypes of genetic abnormalities related to ALS.

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In recent decades neurons derived from induced pluripotent stem cells (iPSCs) have provided ample samples for high throughput screening for neurodegenerative diseases, especially sporadic ALS. Indeed, iPSCs provide functional cells that may be involved in ALS, including neurons, astrocytes, microglia, and skeletal myocytes.

In recent years organoids cultured from patient-derived iPSCs have emerged as a valuable tool in ALS research. Patient-derived iPSCs carry entire genomic information and most of the epigenetic memory of the donors, which provides an unlimited resource to differentiate all tissue types with special interest that may be related to the disease being studied. Because organoids provide a three-dimensional tissue-like architecture, organoids are expected to be a novel model to closely mimic progression of a disease as humanized physiology. Compared to traditionally differentiated motor neurons, organoids contain multiple cell types, including astrocytes, oligodendrocytes, and interneurons, which may contribute to disease development and could be a possible target for treatment.

The US FDA proposed new guidelines on use of organoids as a tool in new drug discovery [8]. However, a large gap exists in using organoids as a tool for drug screening, especially because of organoid heterogeneity and the difficulty in obtaining high-content images. Knowledge of the wide disease models in phenotypic fingerprinting would help apply organoids in evaluating biological effects in drug evaluation. This review summarizes established disease models used in ALS screening, as detailed in **Tables 1** and **2**. Then, we provide insights into the advantages and challenges associated with the application of organoids in high-throughput screening for ALS.

## 2. NON-HUMANIZED MODEL

Various models, including yeast, worms, zebrafish, and mice, have considerable humanized genome homogeneity and accessibility for genetic modification. Such advantages make these models useful tools for exploring the functions of ALS-related genetic mutations. Given the tissue complexity and phenotypes of these animals, scientists could study the functions of genes in contributing to disease development at different scales. Different disease models show their advantages in phenotypic readouts, tissue mirroring, and evaluating approaches (**Figure 1**).

### 2.1 Cellular toxicity model in yeast

The yeast, *Saccharomyces cerevisiae*, is the first fully sequenced eukaryotic single-cell organism [39]. The availability of overexpression or deletion libraries of the entire yeast genome provides a screening system in the identification of ALS targets, especially based on the phenotypes related to general cellular metabolism, such as DNA damage, RNA toxicity, proteinopathies, and mitochondrial dysfunctions [40-43]. Yeast have been

**Table 1** | *In vitro* cellular screening system and utilization in ALS.

| Cell type | Gene of interest | Screening system | Evaluated phenotype | Main discovery | Reference |
|---|---|---|---|---|---|
| Yeast | *ubiquilin-2* | Two-hybrid library with central domain of ubiquilin-2 mutations | Yeast two-hybrid | Interaction of hnRNP with ubiquilin-2 may participate in ALS development | [9] |
| Yeast | *FUS* | FUS truncations | Spotting assays on galactose media | FUS must aggregate in the cytoplasm and bind RNA to confer toxicity in yeast | [10] |
| Yeast | *C9ORF72* | Genome-wide plasmid overexpression screen | Spotting assays on galactose media | Karyopherins and effectors of Ran-mediated nucleocytoplasmic transport participate in dipeptide repeat (DPR)-induced toxicity | [11] |
| Yeast and primary neuron | *TDP-43* | Genome-wide loss-of-function TDP-43 toxicity suppressor screens | Spotting assays on galactose media (yeast); Neural death (primary neurons) | Absence of Dbr1 preventing Dbr1 from interfering with essential cellular RNAs and RNA-binding proteins | [12] |
| K562 cell line and primary neurons | *C9ORF72* | CRISPR-Cas9 whole genome screening | DPR-induced cell death | Tmx2 modifies the *C9ORF72* DPR-induced ER stress response | [13] |
| RPE-1 cell line dual expression $(GGGGCC)_{70}$-EGFP/RFP670 | *C9ORF72* | CRISPR-Cas9 whole genome screening | FACS-based EGFP/RFP flow cell sorting | DDX3X is a modifier of non-AUG DPR protein production | [14] |
| Induced neurons from human iPSCs | *N/A* | CRISPR-Cas9 whole genome screening | Neural survival | Lysosomal protein prosaposin modifies neural-specific response to oxidative stress. | [15] |

**Table 2** | *In vivo* screening system for ALS.

| Species | Gene of interest | Screening system | Evaluated phenotype | Main discovery | Reference: |
|---|---|---|---|---|---|
| *Drosophila* | *C9ORF72* | GGGGCC (G4C2) repeat expansion in *C9ORF72* | Toxicity<br>Larval size<br>Synaptic bouton number<br>Crawling ability | The G4C2-58 repeat hinders nucleocytoplasmic transport through the nuclear pore. | [16] |
| *Drosophila* | *C9ORF72* | G4C2 repeat expansions in *C9ORF72* | Eye degeneration | C9ORF72 affects retrograde Golgi-to-ER vesicle-mediated transport. | [17] |
| *Drosophila* | *TDP-43* | TDP-43$^{G298S}$ mutant | Global metabolomics profiling<br>Motor function | TDP-43 proteinopathy increases the TCA cycle and levels of glutamine, while decreasing dopamine secretion. | [18] |
| *Drosophila* | *FUS* | Human mutant *FUS* transgenes | Eclosion rate<br>Loss of CCAP neurons | Downregulation of nucleoporin 154 and exportin 1 (XPO1) prevents FUS-induced neurotoxicity. | [19] |
| *Drosophila* | *SOD1* | SOD1$^{H71Y}$ mutant | Motor function axon pathology | The locomotor defects and paralysis caused by the dsod1 mutation and the reduction in arborization and loss of markers in the MN-I2 motor neuron are both age-related | [20] |
| *Caenorhabditis* | *C9ORF72* | G4C2 repeat expansions in *C9ORF72* | Developmental stage<br>Brood sizes<br>Motor function<br>Lifespan | There may be excessive activation of lysosomal activity in ALS. | [21] |
| *Caenorhabditis* | *alfa-1* (the ortholog of *C9ORF72*) | *alfa-1* mutation | Motor function<br>GABAergic motor neuron degeneration | Loss of alfa-1 leads to degeneration of GABAergic motor neurons and increased stress sensitivity.<br>Deletion of alfa-1 enhances motor defects by mutant TDP-43. | [22] |
| *Caenorhabditis* | *SOD1* | *SOD1* G85R mutant | Motor function | Misfolded SOD1 affects the biogenesis and/or transport of synaptic vesicles. | [23] |
| *Caenorhabditis* | *SOD1* | *SOD1* G85R mutant | Glutamatergic neuron degeneration | Figo-1 and sod-1 may lie in the same genetic pathway. | [24] |
| *Caenorhabditis* | *TDP-1 (TDP-43)* | CRISPR-Cas9-based genome editing *tdp-1* | Neurodegeneration | Yielded a non-ambiguous null allele for tdp-1 and a humanized hTARDBP. | [25] |
| *Caenorhabditis* | *TDP-1 (TDP-43)* | Mutant alanine53 threonine (A53T) human a-synuclein | Movement<br>Development<br>Lifespan<br>Protein aggregates<br>Neuronal pathology | TDP-1 supports the expression of α-synuclein. | [26, 27] |
| *Caenorhabditis* | *FUS* | *FUS* C-terminal deletion | Electrophysiology<br>Synaptic structure and function | FUS disrupts the organization and docking of vesicles at the NMJ. | [28] |
| *Danio* | *SOD1* | SOD1 G93R mutant | Locomotor activity | 1-Fe potentially impacts the progression of ALS and degeneration of motor neurons. | [29] |
| *Danio* | *SOD1* | Injected human SOD1$^{A4V}$ mRNA | Larvae movement<br>Motor axon length | Injection of hSOD1 mRNA leads to shortening and abnormal branching of motor axons, resulting in a reduction in the locomotor distance. | [30] |

**Table 2** | Continued

| Species | Gene of interest | Screening system | Evaluated phenotype | Main discovery | Reference: |
|---|---|---|---|---|---|
| *Danio* | *TDP-43* | *TDP-43* double mutant | Morphology<br>Coiling<br>Locomotor activity<br>Synapes<br>NMJ structure | Generated tardbp–/– and tardbpl–/– double mutants highlight the importance of partial and complete loss of function of TDP-43 isoforms in the zebrafish functional impairment model. | [31] |
| *Danio* | *FUS* | *FUS* homologous gene deletion | Lifespan<br>Locomotor<br>Neuron length<br>NMJ | Cell damage is associated with dysregulation of acetylcholine receptor (AChR) subunits and histone deacetylase 4 mRNA expression.<br>The link between the altered function of FUS and tau proteins may play a role in the development of tauopathies. | [32] |
| *Danio* | *C9ORF72* | G4C2 repeat expansions in *C9ORF72* | Early mortality<br>Locomotor activity<br>Muscle<br>Motor neuron<br>ALS-like behavioral phenotypes | C9ORF72 expansion activates the heat shock response (HSR) in zebrafish.<br>The combination of ivacaftor and lurasidone was shown to reduce HSR activation. | [33] |
| *Mus* | *SOD1* | Low copy number of SOD1-G93A mutants | Posture and range of motion<br>Survival<br>Video fluoroscopic swallow study (VFSS)<br>Denervation atrophy of genioglossus | Significant tongue denervation atrophy in hindlimb and mixed phenotype LCN-SOD1 mice. | [34] |
| *Mus* | *TDP-43* | *TDP-43* overexpression | Motor neuron number<br>NMJ denervation status<br>Terminal Schwann cell morphology | This mouse model represents a severe ALS phenotype, including NMJ pathology, distal muscle denervation, and motor neuron loss. | [35] |
| *Mus* | *FUS* | Conditional knock in *FUS* | Neuron apoptosis<br>GABAergic synapses | The synaptic FUS RNA targets encode essential protein components for synapses. | [36, 37] |
| ***Mus*** | *C9ORF72* | G4C2 repeat expansions in *C9ORF72* | Paralysis<br>Anxiety-like behavior<br>Survival<br>Neurodegeneration | Established a new BAC transgenic mouse model with ALS/FTD phenotype. | [38] |



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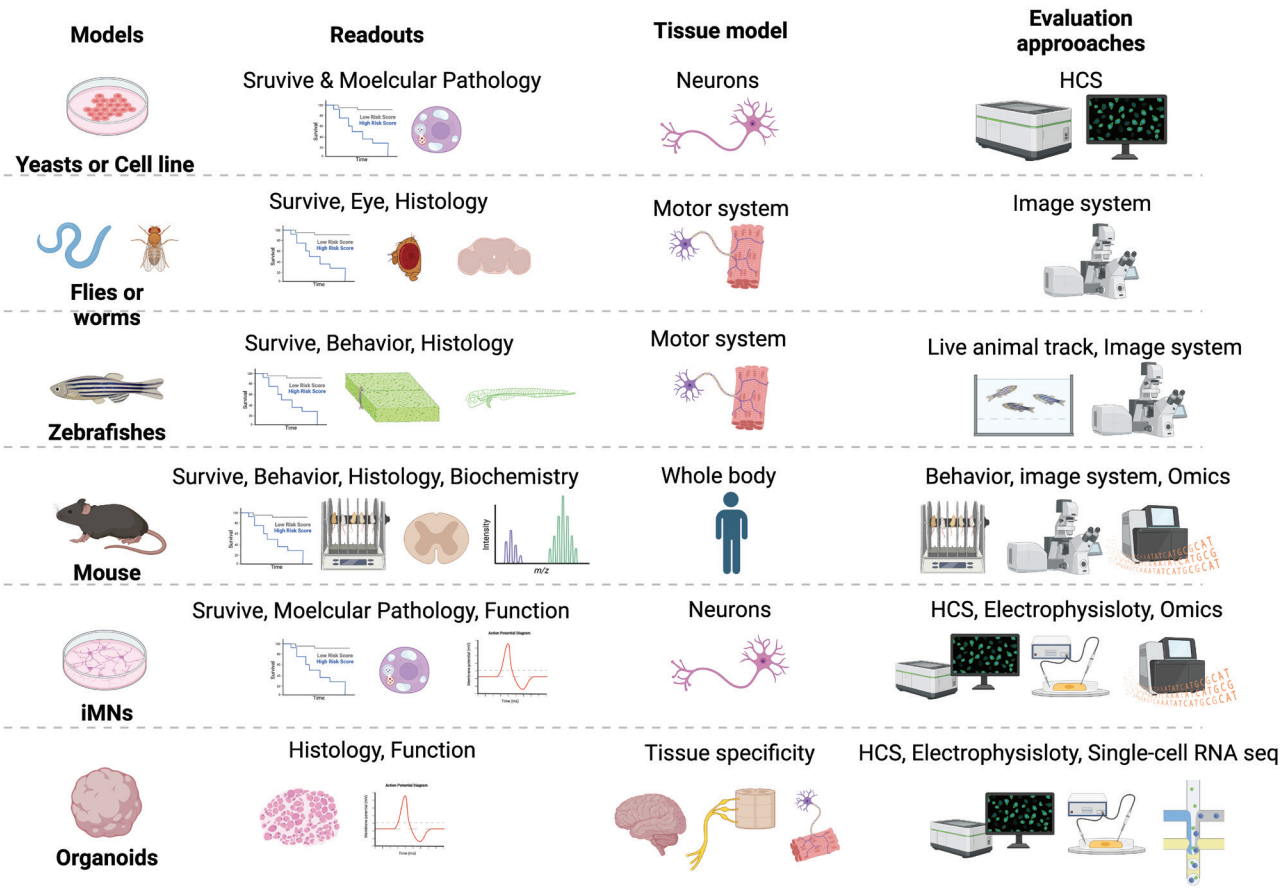


**Figure 1 | Summary of the ALS disease model for drug evaluation.**
The diagram shows the different disease models of ALS as well as the application in ALS drug exploration. The phenotypes serve as the evaluating readout for tissue or organ specificity and the devices for evaluating are listed for each model type.

applied as an ALS model via gene editing, which focuses on cellular pathologies as the readout. The expression of *FUS* in yeast recapitulates several pathologic features of ALS, such as nuclear-to-cytoplasmic translocation, formation of cytoplasmic inclusions, and cytotoxicity [44]. Researchers have currently identified 37 human genes that suppress FUS-induced toxicity from the screening library containing 13,570 human genes cloned in an inducible yeast-expression vector [44].

## 2.2 Neurodegenerative model in Drosophila melanogaster and Caenorhabditis elegans

The short life span and the relatively simple nervous system in *D. melanogaster* and *C. elegans* make *D. melanogaster* and *C. elegans* ideal models to mimic ALS-associated neurodegeneration. *D. melanogaster* has 14,000 genes, approximately 40% of which have homology for 75% of human genes with functional orthologues [45]. The tools for genetic screens could be created by crossing wild-type flies to deletion stocks or using RNAi lines, which cover approximately 90% of the genome and are publicly available [46]. Neurologic phenotypes, including climbing and crawling, brain histologic profiles, eye morphologies, electrophysiology, eclosion rate, and lifespan could be used for mirroring ALS-associated clinical symptoms [47]. Eyes are the most widely used tissue for evaluating modifier screening because testing is relatively easy [48]. Transgene expression in flies is usually achieved using the bipartite Gal4-upstream activating sequence (UAS) system derived from gene expression for galactose metabolism in yeast. The tissue marker of the fly is detectable using UAS-β-galactosidase (UAS-lacZ) or UAS-green fluorescent protein (GFP). This detectable tissue marker could be used for assessing the tissue-specific effects of specific gene knockdown using UAS-RNAi lines [http://www.flyrnai.org/DRSC-OVR.html] [49].

There are 19,000 genes in *C. elegans* based on gene sequencing results [50]. Several well-established models, such as *C9ORF72* [51], *SOD1* [24], *TDP43* [52], and *FUS* [53], have been extensively utilized based on the specific phenotypes, which mainly focus on lifespan, motor function, and neurodegeneration. The survival rate, the integrity of neuromuscular junctions, and cellular pathologies

could be applied as the readout of ALS in *C. elegans*. Recent research suggests that metformin enhances autophagy and extends lifespan through the daf-16 pathway in human *SOD1* (Hsod1) transgenic worms [54]. Ectopic expression of ALS-associated human FUS impairs synaptic vesicle docking at neuromuscular junctions in *C. elegans*, which has led to the emergence of a population of large, electron-dense, and filament-filled endosomes [28].

### 2.3 Spinal motor degenerative model in zebrafish

Zebrafish have become the most applicable vertebrate model for drug screening due to the high degree of conservation with human genes, ease of breeding, and accessibility of genetic modification. A comprehensive sequencing study has shown that zebrafish have at least one homologous gene with >70% of human genes, including many at-risk genes in human neurodegenerative diseases [55]. The small size and transparent profile of the zebrafish make zebrafish more suitable for high-throughput screening, especially in confirmation of the disease phenotype.

The behavioral testing system of zebrafish is also mature for studying ALS symptoms, which mainly focuses on the motor system. By testing the traveled distance and motor axon length of zebrafish injected with human *SOD1A4V* mutant mRNA, Robinson et al. [30] provided the first evidence of a correlation between the motor axonopathy of *SOD1* mutations in zebrafish and motor impairments. By injecting *SOD1* mutant mRNA into zebrafish embryos, DuVal et al. [56] investigated the role of the W32 residue in *SOD1* cytotoxicity. DuVal et al. [56] reported that the nucleoside, telbivudine, exerts rescue effects by targeting W32. Shaw et al. [33] constructed the first C9-ALS/frontotemporal dementia (FTD) zebrafish model that recapitulates the motor defects, cognitive impairment, muscle atrophy, motor neuron loss, and death features observed in early adulthood in human *C9ORF72*-ALS/FTD and showed that the compounds, ivermectin and riluzole, reduce activation of the heat shock response induced by C9ORF72- hexanucleotide-repeat expansion (HRE).

### 2.4 Mammalian ALS model in mice

Mice serve as the mammalian rodent disease model for most neurodegenerative diseases, including ALS. The advantage of mouse models relies on mimicking the clinical-related phenotypes of ALS in detail, such as symptoms in the bulbar region of the brains and limbs.

The mouse model covers all the anatomic structures of humans for drug evaluation and provides a systematic tool for testing the effects of the disease at the level of multiple tissue interactions directly and indirectly. The interactions include the cell network inside the brain or spinal cord (e.g. neuron-neuron interactions and neuron-glia interactions) and the factors beyond neural tissue (e.g., liver-brain axis, gut-brain axis, and immune-brain/spinal cord interactions), which are considered to play a role in the development of ALS [57-59].

## 3. HUMAN-DERIVED DISEASE MODEL

The non-human animal model is limited in drug development, especially diseases like ALS, which show a complex contribution between genetic and epigenetic factors. Most investigational drugs in late-stage clinical trials are the lack of effectiveness (57%) and safety concerns [17%] [60]. Neurodegenerative diseases, such as ALS and Alzheimer's disease (AD), are mostly sporadic with complex etiologies, which makes recapitulating the phenotypes of these diseases a challenge [61, 62]. Therefore, a new disease model with cell diversity derived from patients with high-throughput and disease-specific features is expected to bridge the gap between the mouse model and ALS drug screening. Patient-derived iPSC organoids may be a next-generation tool for ALS modeling and drug evaluation.

### 3.1 Cell lines and neurons

Cultured cell lines or primary neurons offer valuable mammalian *in vitro* systems for drug screening. Immortalized cell lines can provide sufficient samples and ensure comprehensive coverage of screening libraries. Screen strategies of the cell lines for ALS therapy mainly depend on the molecular pathways associated with the disease and are mainly applied in whole genome screening. CRISPR screening systems offer a powerful tool for drug discovery across various applications, including ALS [63]. Kramer et al. [13] conducted a comprehensive whole-genome CRISPR-Cas9 screen on the K562 cell line with hits, including polyGR/polyPR, a presentative gain of functional dipeptide repeat proteins (DPRs) from the HRE within the C9ORF72 gene. By using cytometry cell sorting for the retinal pigment epithelium (RPE)-1 cell line carrying RFP670 expressed in the AUG translation as an internal control, Cheng et al. [14] conducted a genome-wide CRISPR-Cas9 by fused $(GGGCC)_{70}$ repeats conjunct with EGFP in the GA frame. To avoid genetic KO-induced neural development interference, Tian et al. [15] established the genome-wide CRISPRi (interference) and CRISPRa (activation) systems to perform the genome screens directly in human neurons, which avoided interference from the neural developmental stage. Based on stress granules, Fang et al. [64] performed the screen on HEK293 and induced motor neurons with the neuroprotective small molecule libraries.

Patient-derived iPSC motor neurons duplicate the survival vulnerability of neurons in patients compared to cell lines, which were utilized as the readout for drug screening. Using induced motor neurons (iMNs) from C9ORF72-HRE ALS patients, Shi et al. [65] reported a *C9ORF72* haploinsufficiency, known as a C9ORF72-HRE loss of function (LOF), which mediated neurotoxicity. Such a LOF could be resorted by treatment of the RAB5 chemical modulators through screening with a small molecular chemical library [EMD Millipore kinase collection and Stemselect library, 3.3 μM final concentration]

[65]. In 2023 the same research team identified the general iMN phenotypes from *C9ORF72*, *TDP43*, and sporadic ALS. With a chemical library screening (Microsource Spectrum Collection, which is composed of approved drugs, bioactive compounds, and natural products), a SYF2 inhibitor was discovered that attenuated the iMN degeneration process [66]. By screening the phenotypes, including neurite length, LDH release, stress granules, and cleaved caspase-3 protein accumulation, Fujimori et al. [67] confirmed the efficacy of the Parkinson's disease drug, ropinirole, in protecting ALS motor neurons. In the phase 1/2a clinical study of the open-label extension period, the ropinirole group presented significant suppression of ALS functional rating scale–revised (ALSFRS-R) decline with an additional 27.9 weeks of disease progression-free survival [68]. To date, the Answer ALS project has established the patient-derived iPSCs and collected the relative clinical data, whole-genome sequencing data, RNA-seq, ATAC-seq, and iMN proteomic data from the iPSCs [69, 70].

## 4. ORGANOIDS: NEXT GENERATION IN DRUG DEVELOPMENT FOR ALS

Organoids have advantages in studying diseases within the scope of neurodegeneration by duplicating cell diversity in the tissue-like architecture, especially the variant disease-associated phenotypes compared with 2D neurons. Lancaster et al. [71] developed the first brain organoids from human iPSCs, which paved the way for a new era of disease modeling in neurologic disorders. Using iPSCs or adult stem cells as a resource, 3D culture techniques enable the generation of 3D tissue spheroids in mimicking tissue organization and functions, which provides a new model in developmental biology, disease modeling, biobanking, and multiple omics analysis. Increased cell diversity of organoids in 3D structure provides the human tissue-like microenvironment compared to 2D-induced neurons for disease duplication. For example, PIEZO channels sense extracellular mechanical stimuli to modulate stem cell functions that can be duplicated in the intestine organoid system [72]. Organoids have been utilized to establish numerous systems for disease modeling, including brain [73], hepatic [74, 75], and lung and colonic organoids [76].

Considering the upper and lower motor neuron defects in ALS, organoids mimic the brain, spinal cord, and neuromuscular system and are considered dominant tools for drug testing (**Table 3** and **Figure 2**). ALS-associated phenotypes have been reported in the above-mentioned organoids from patient-derived iPSCs. Astrocytic and neuronal protein stasis and DNA repair dysregulation have been observed from brain organoid slices of individuals with C9ORF72 ALS/FTD [80]. Pereira et al. [77] developed sensorimotor organoids containing motor neurons, sensory neurons, skeletal myocytes, astrocytes, and microglia, as well as the vascular system. Such organoids derived from ALS patient-derived iPSCs have been shown to have significant defects in muscular contraction [77]. Injection of ALS patient spinal cord extracts containing TDP-43 in healthy brain organoids could result in pathogenic TDP-43-induced cell apoptosis and astrogliosis, as well as prion-like spread of TDP-43 in the human central nervous system [78]. Szebényi et al. [80] generated sliced brain organoids induced from the iPSCs of C9ORF72 patients, which represented the early astrocytic ALS/FTD pathologic features, including aggregating DPRs, DNA damage, and over-autophagy. Our team generated the neuromuscular organoids (NMOs) and duplicated the C9ORF72-related neuromuscular

**Table 3** | Organoid modeling ALS and neurodegeneration.

| Types | Gene of interest | Induction origin | Readouts | Main discovery | Reference |
|---|---|---|---|---|---|
| Sensorimotor organoids | *C9ORF72*<br>*FUS* | Neuromesodermal progenitors | NMJ phenotypes<br>Astrocytes<br>Microglia<br>Vasculature | Generated sensorimotor organoids modeling ALS subgroups and identifying cellular impairments at neuromuscular junctions. | [77] |
| Cerebral organoids | *TDP-43* | Ectodermal progenitors | TDP-43 pathology<br>Astrocyte proliferation<br>Cellular apoptosis | Used human cerebral organoids to validate TDP-43 propagation in ALS. | [78] |
| Neuromuscular organoids | *C9ORF72* | Neuromesodermal progenitors | Muscular contraction weakness<br>NMJ integrity downregulation<br>DPR aggregation<br>Electrophysiologic properties | Discovered the aggregated DPRs in astrocytes and neurons.<br>A contraction weakness in muscular sides may manifest earlier that histologic downregulation of NMJs. | [79] |
| Sliced cerebral organoids | *C9ORF72* | Ectodermal progenitors | Neural network function<br>Cell homeostasis<br>ER stress and astroglia<br>DNA damage | Revealing an increase in autophagy signaling protein P62 levels and DNA damage in astrocytes, which were improved by GSK2606414 treatment. | [80] |

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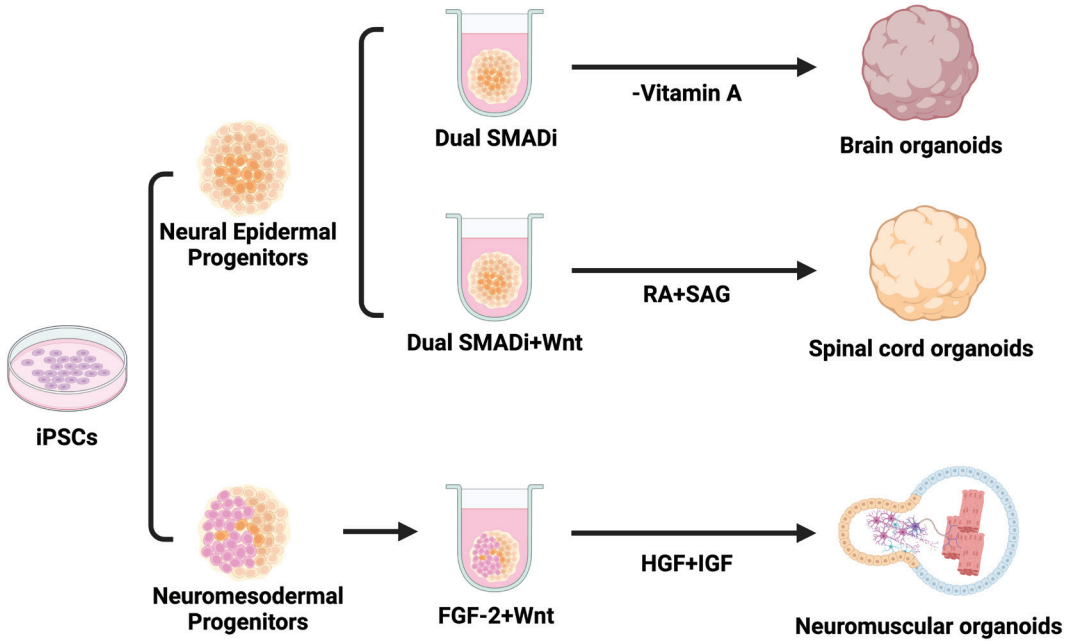


**Figure 2 | Main organoid types mimicking ALS pathologies.**
The diagram shows the induction pathway and the main types of organoids for ALS evaluation, including brain, spinal cord, and neuromuscular organoids.

decline, as well as the trunk spinal pathologies based on the iPSCs from the same mutation [79]. As noted, the two systems had a positive response to the PERK inhibitor, GSK2606414, indicating the application of organoids in drug tests. With respect to neural functional analysis, the multiple electrode array (MEA) approach has been used in electrophysiologic testing in brain and neuromuscular organoids [79, 80].

Undoubtedly, organoids derived from patient-derived iPSCs resolved the limit of the clinical sample and the throughput of the drug screening for ALS. The *in vitro* response of patient-derived iPSC organoids can predict the response of patients to treatment thereby opening up new opportunities for drug discovery [81, 82]. However, organoid variants for benchmarking requires further confirmation to apply organoids in high content screening.

## 5. OPTIMIZING ORGANOID CULTURES FOR ALS MODELING

A model system for drug screening requires stable and reproducible phenotypes, which could reflect the key biological underpinning of ALS. For *in vitro* systems, especially induced motor neurons, up- and down-stream technologies ensure the considerable number of motor neuron production fulfilling the high-content screening via biomarker imaging.

The phenotypic analysis for organoids is currently limited by heterogeneity, which comes from the individual sample, batch-to-batch, and different iPSC donors. Optimizing iPSCs further into the native status could be an option to improve organoid quality. By comparing the line-to-line variance and within-line variance of sensorimotor organoids between non-isogenic iPSCs from healthy or ALS patients and the isogenic iPSCs after ALS-related gene modification (e.g., *SOD1, TDP43*, and *PFN1*), Pereira et al. [77] reported that the isogenic line among line variance and within-line variance was significantly lower than the non-isogenic group, which from the iPSC clone pick up during genetic modification. Such accumulating variance during iPSC replication may increase heterogeneity among organoids from different individuals. Reprogramming of donor cells independent of Yamanaka factors may provide an alternative method to optimize iPSC quality, such as chemical reprogramming and expanding pluripotent stem cells [83, 84]. Transient-naive-treatment (TNT) protocol methods may provide an updating resource of iPSCs for organoid generation [83, 85]. Additionally, suppliers of the chemicals, growth factors, and basal media during organoid inductions are factors that cannot be ignored in the stability of organoids. To overcome such current issues, technical and differentiation batch repeats are required for utilizing organoids in drug screening and disease modeling.

The human-like biological clock is another concern for organoids in mimicking degenerative disorders. Most organoids typically represent early fetal development, even after 50 days from the beginning of induction. Such a long-term culture for organoids mostly requires an orbital shaker to ensure the supply

of oxygen and nutrition [71, 79, 80]. With respect to brain organoids, the time of phenotypic significance, including autophagy, DNA damage, and aggregation of the DPRs, was reported around day 150 [80]. For neuromuscular organoids system, the same iPSCs from C9ORF72 donors showed an early contraction defect on day 50 and remarkable degenerative neuromuscular junctions and aggregation of DPRs on day 100 [79]. Those lines of evidence indicate a distinctive time of disease progress in organoids compared to real *in vivo* conditions. One of the reasons for this finding might be the underlying difference in cell diversity between organoids and *in vivo* conditions. Lack of cell diversity, such as microglia and vascular epithelial cells, may result in accelerated development of disease phenotypes in organoids [86, 87].

The sheer stress during shaking may be another factor in the variation of organoids during morphogenesis, which may be reflected in the random shape and brain regions of the cortical organoids in long-term culture. Development of an auto-bioreactor system to stabilize the sheer force during the long-term culture phase is widely considered necessary for mimicking age-related neurodegenerative disease. Qian et al. [88] developed a bioreactor named spin omega for brain region-specific organoids, which showed advantages in stabilizing sheer force affection and demonstrated application for Zika virus evaluation. To address the issue of an insufficient nutrient supply and inadequate diffusion of nutrients, Giandomenico et al. [89] generated cerebral organoid slices at an air-liquid interface and demonstrated improved neuronal survival and axonal growth. Such models have been utilized in evaluating the disease phenotypes of C9ORF72-HRE-induced FTD/ALS pathology [80]. Supported by the spin omega device, Qian et al. [90] also developed sliced brain organoids and successfully addressed the fundamental limitation that has hindered the accurate replication of the architectural features of late-stage human cortical development in cortical organoids. In addition to those methods for optimization, manual picking up organoids with standard guidelines provides a relatively applicable method for evaluation. In our research involving neuromuscular organoids modeling, the organoids with one bright and one dark side, the olive shape was picked for assessing the disease relative phenotype [79].

Niche factors, including the elasticity and biochemical support from the extracellular matrix (ECM), are also considered key regulators for optimizing tissue stability and development efficiency of organoids [91]. Co-culture systems enable organoids to mimic the interaction of peripheral cells with neural systems, such as microglia [92]. Embedding organoids into an artificial hydrogel containing essential ECM components supplies the physical and biochemical environments to the disease model [93]. Assembloids and organ chips are developed for further obtaining the multiple connective or distal organ interactions. Fusing organoids from different brain regions successfully mimic the neural network between dorsal and ventral regions or from the cortex to spinal cord [94, 95]. Further integration of different organoids into microfluid-derived chips could be used for studying the distal organ interaction with the brain or spinal cord, which would further reflect the systematic pathologies of ALS [96]. The above-mentioned approaches would help promote the stability of organoids and expand the tissue complex of the organoids for mimicking more biological mechanisms of ALS (**Figure 3**).

## 6. SET READOUTS OF ORGANOIDS ACCORDING TO HIGH-THROUGHPUT SCREENING

Several works have been initiated to use organoids as the model for high-throughput analysis, which has paved the way for organoids in ALS drug screening [97]. By integrating mathematical modeling with cerebral organoid (iCO) phenotypic analysis, Park et al. [98] utilized 1300 organoids from 11 participants with sporadic AD and established a high-content screening (HCS) system for testing FDA-approved blood-brain barrier permeable drugs. Park et al. [98] manually picked the organoids and performed the transparency for facilitating HCS analysis. The factors containing biomarker area, shape, and diameter of the organoids were included for automatic quality control (QC). Due to the difficulties with organoid 3D shape, the tissue transparency technique may provide the advantage necessary for organoids based HCS. By utilizing the benzyl alcohol/benzyl benzoate (BABB)-based clearing protocol, Renner et al. [99] developed a fully automated high-throughput workflow for 3D-based chemical screening for human midbrain organoids. As an alternative strategy, several teams transferred attention to generating Matrigel-supported 2D organoids to avoid difficulties in 3D imaging. As mentioned above, the sensorimotor organoid induced by neuromesodermal progenitors was cultured in the Matrigel and established the motor neurons and skeletal muscle tissue [77]. By optimizing somitogenesis with dual SMAD inhibitors, Urzi et al. [100] developed self-organized neuromuscular junction organoids in a 2D cultural surface, which could duplicate spinal muscular atrophy-associated pathologies induced by patient-derived iPSCs. Apart from image analysis, the level of biochemical markers, including lactate dehydrogenase (LDH), neurofilament light chain (NfL), and redox oxygen species (ROS), were reported as the readouts for screening [70, 101, 102]. Multiple disease-associated phenotypes, such as neurite length, LDH release, cleaved caspase-3, and G3BP stress granules, were combined for high-throughput screening in iMNs [67]. Moreover, setting one key readout for narrowing down the candidates with multiple stages of testing would be another strategy for screening [66, 97].

To avoid the interference of technicians for drug screening, a hardware device for automated laboratories

Acta Materia Medica

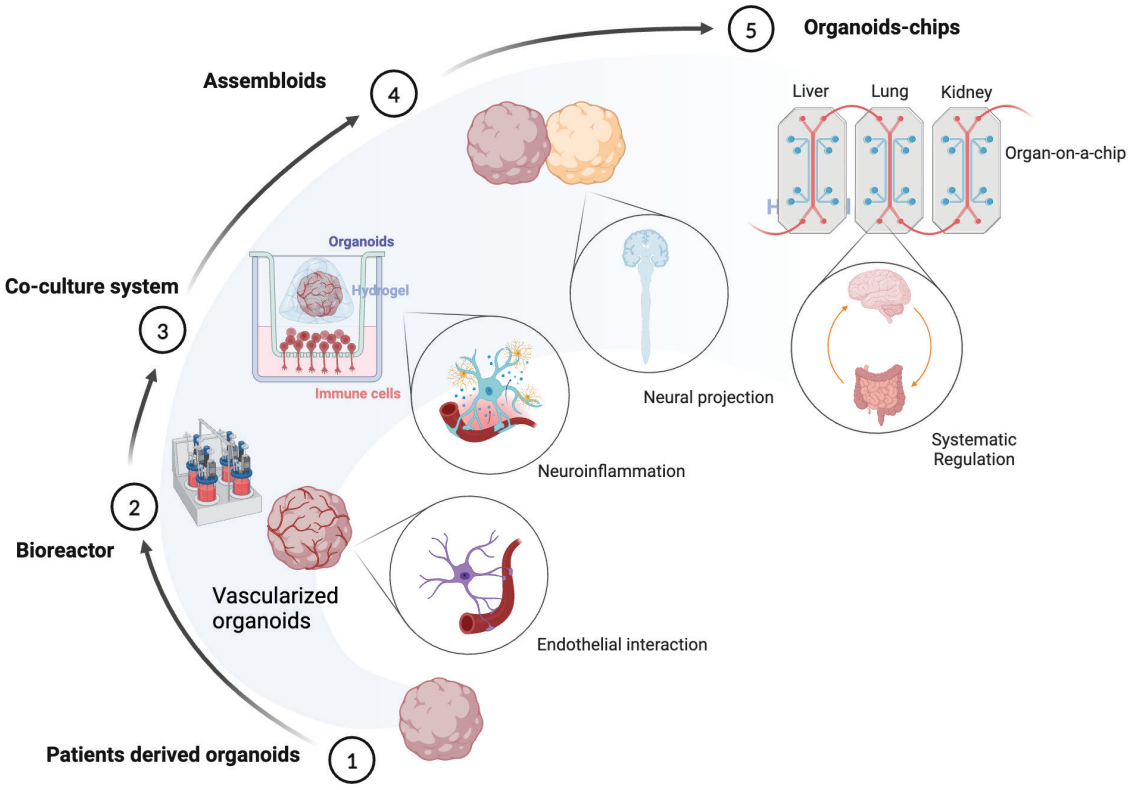


**Figure 3 | Optimizing the organoids mimicking disease.**
The diagram shows the step-by-step methods to optimize organoids for duplicating the tissue complex with ALS as the target. Bioreactor devices, co-culture systems, assembly technologies, and organoid chips were utilized to produce organoid systems with vascularization, immune responses, neural projection, and distal organ interactions.

is the essential step for high-throughput screening, especially for industry. An automated platform combination with a liquid handler, acoustic droplet ejector, robotic arm, and cell counter devices was designed to culture midbrain-specific organoids for high-throughput drug screening [103]. Deep learning-based categorizing helped build the phenotypic fingerprints of the organoids for further evaluating the unidentified drug effects. By treatment with identified targeting chemicals, Lukonin et al. [104] generated multivariate feature profiles for hundreds of thousands of intestinal organoids to quantitatively describe the phenotypic landscape and used phenotypic fingerprints to infer regulatory genes. It is expected that organoids will be utilized for ALS drug screening with the development of big data analysis, RNA sequencing, and phenotypic screening. Considering the current issue of the instability of organoids, drug screening could be performed in separated steps or based on combined phenotypic landscapes in a single set of organoids (**Figure 4**). Primary screening would focus on using a single model, including iMNs or organoids, after quality control. The priority readouts are required with the direct phenotype in association with motor neuron protection. For secondary screening, narrowing down libraries are processed in multiple organoids from iPSCs of different donor patients. A systematic score of the drug effects by combining the multiple phenotypes of the organoids with the RNA profile may help to determine a final drug candidate for preclinical purposes.

## 7. CONCLUSION AND PROSPECT

Genetic and epigenetic memory of iPSCs enable the capacity of organoids in disease modeling, especially based on individual patients. Further development of 3D high speed and high content images with an artificial intelligence trajectory for ALS may further enhance organoids as a tool for drug screening. Moreover, models for ALS study can cover different aspects that are far more than the above-mentioned models in this review. *In vivo* mammalian models, including rodents and non-human primates, also show a powerful potential for disease modeling and drug development. Moreover, systematic organoid tools, particularly "human on a chip," which organically combines organoids from different systems together on a micro-fluid chip, also exhibit a great advantage in humanized drug evaluation.

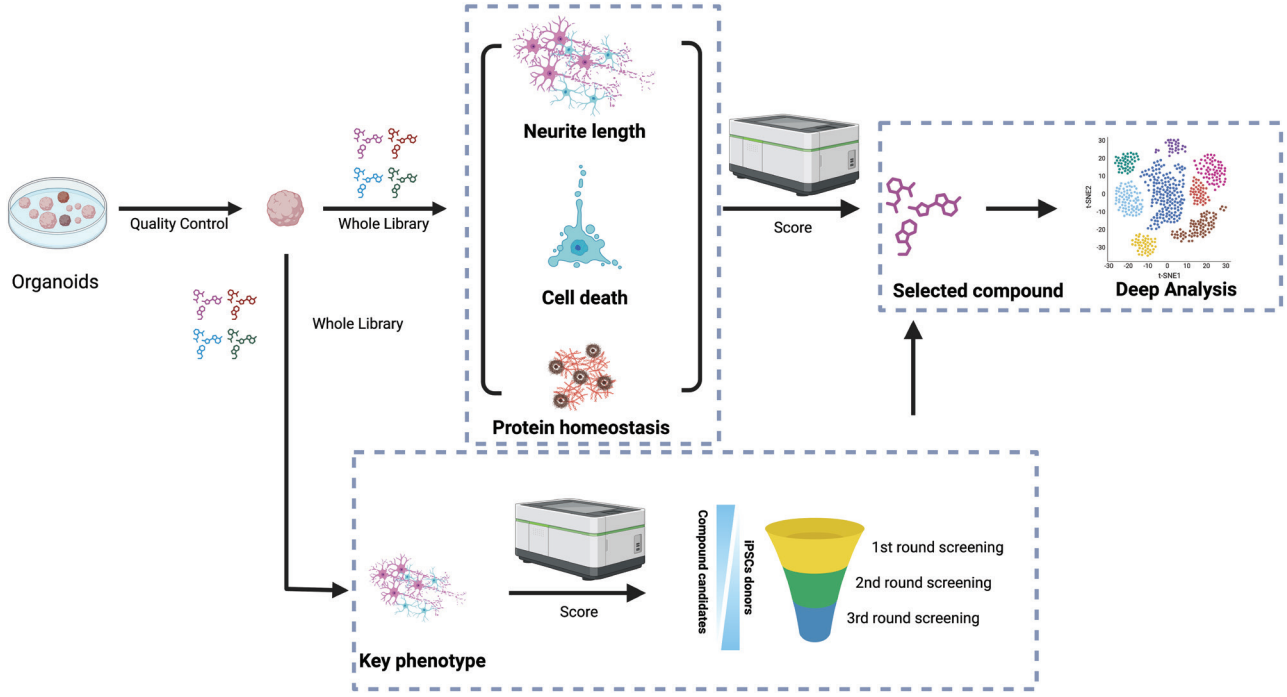


**Figure 4 | Analysis methods using organoids as a tool for ALS drug screening.**
The workflow chart shows the strategies for drug screening via patient-derived organoids. Combined neurodegenerative phenotypic fingerprints or multiple rounds of the screening given single phenotypes may be applied for high-throughput screening. Deep analysis, such as single cell omics studies, will be further used for exploring the mechanisms underlying select candidates from screening.

Patient-derived iPSC biobanking provides the resource of disease modeling via multiple types of organoids for studying ALS. The Answer ALS project initiated from Cedars-Sinai Hospital has collected sufficient resources of ALS patients with iPSCs and various genotypes and sporadic cases [69]. Combined with different organoids, co-culturing with immune cells or embedding the organoids into bio-engineered hydrogels would provide a tissue-specific microenvironment. Further building of the assembloids and co-culturing different organoids into chips would provide human-like organ interaction as well as the distribution of the drugs nearby and distally. Finally, the tested drug would be further investigated in animal and human samples (**Figure 3**). With the development of high-content image technology, artificial intelligence assisting phenotypic landscape analysis, and standardization of 3D cultures, organoids may become the dominant model for drug development and target screening for ALS.

## ABBREVIATIONS

ALS: amyotrophic lateral sclerosis; iPSCs: induced pluripotent stem cells; FUS: fused in sarcoma; DPRs: dipeptide repeats; DEPseq: deep sequencing; ER: endoplasmic reticulum; RPE-1: retinal pigment epithelium; FACS: fluorescence-activated cell sorting; KO: knock out; UAS: upstream activating sequence; NNDs: neurodegenerative diseases; ALSFRS-R: ALS functional rating scale–revised.

## ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA

Not applicable.

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## CONFLICTS OF INTEREST

All listed authors have no conflicts of interest.

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