A look into the future of ALS research

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Although amyotrophic lateral sclerosis (ALS), also referred as ‘Lou Gehrig’s Disease,’ was first described in 1869 and the first disease-associated gene was discovered almost 20 years ago, the disease etiology is still not fully understood and treatment options are limited to one drug approved by the US Food and Drug Administration (FDA). The slow translational progress suggests that current research models are not ideal to study such a complicated disease and need to be re-examined. Progress will require greater insight into human genes and biology involved in ALS susceptibility, as well as a deeper understanding of disease phenotype at the histological and molecular levels. Improving human disease outcome will require directing focus toward improved assessment technologies and innovative approaches.

Introduction

Since the first gene associated with ALS was identified almost 20 years ago, researchers have relied primarily on animal models to study the mechanisms of disease progression and to identify therapies. These studies, funded mainly by the ALS Association and the National Institute of Health (http://report.nih.gov/categorical_spending.aspx), have cost nearly US$700 million over the past 10 years in the USA alone. One of the main focuses has been on recapitulating the human disease in animal models, which has resulted in the identification of a single drug, riluzole, which was approved by the FDA for ALS treatment in 1995. However, the benefits of riluzole are limited to extending the lifespan or time to tracheostomy by an average of 3 months. Over the past decade, investigational new drug (IND) applications based on data collected using animal models of ALS have resulted in 11 human clinical trials, all of which failed to demonstrate efficacy [1]. In fact, some drugs, which effectively slowed disease progression in mice, resulted in accelerating the progression in humans [2]. The repeated failure of drug translation from animal models to humans seen with ALS is disappointing in terms of financial and, more importantly, human costs. Here, we review the current models used in ALS research and suggest a re-examination of the field to focus the research on more human-based approaches.

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ALS

ALS is a progressive, fatal neurodegenerative disease characterized by loss of motor neurons in the brain and spinal cord. According to the National ALS Registry (http://www.cdc.gov/mmwr/preview/mmwrhtml/s6307a1.htm), 12,000 people in the USA have a definite diagnosis of ALS, for a prevalence of 3.9 cases per 100,000 persons. The disease is more common among white males, non-Hispanics and persons aged 60–69 years. Between 90% and 95% of these individuals develop a sporadic form of ALS (sALS), occurring without known genetic risk factors, and have no family history of the disease. The remaining 5–10% of cases are inherited and classified as familial ALS (fALS), which is associated with more than a dozen genes. Both recessive and dominant forms of ALS have been identified, but most fALS is passed on in an autosomal-dominant manner.

ALS is a complex disorder with a spectrum of phenotypes that could comprise a single disease or represent several closely related disorders with different causes but similar clinical manifestations. The clinical progression is generally rapid, beginning with muscle loss followed by muscle degeneration, paralysis, and respiratory problems. Most patients succumb to the disease within 3–5 years after diagnosis because of respiratory or cardiac deficits. Although motor function declines, sensory, cognitive, and emotional capabilities are generally left intact. Although the etiology underlying ALS progression is unknown, various cellular populations and processes are known to be involved. Key pathological features include motor neuron loss (lesions), retraction of motor neuron axons from neuromuscular junctions, the appearance of inclusion bodies within neurons and astrocytes, ubiquitin-positive protein aggregates in neurons, and blood–brain barrier disruption. Although many gene mutations have been shown to be associated with the development of fALS, a genetic contribution to sALS is still unclear. Risk factors for sALS include gender [3], exposure to toxic chemicals [4,5], and trauma experienced during military service [6].

The first study associating a genetic association in ALS, specifically Cu/Zn superoxide dismutase (SOD1), was published in 1993 [7]. SOD1 is a ubiquitous protein thought to be responsible for protecting cells from oxidative stress by neutralizing cytoplasmic free radicals. SOD1 is a soluble protein located in the nucleus, cytosol, mitochondria, and peroxisomes, where it converts superoxide radicals to oxygen and hydrogen peroxide. Knockout and functional studies indicated that most mutations in SOD1 result in gain of toxic function by destabilizing the protein and causing it to fold into a non-native, harmful conformation [8]. Current theories suggest that these misfolded proteins produce a toxic protein aggregate buildup [9], although the complete mechanisms of disease progression are unknown. Mutations in SOD1 account for 20% of fALS and 1–3% of sALS cases. Different mutations in SOD1 also have penetrance that can vary depending on factors such as age, sex, or ethnicity [10].

From 2001 to 2011, mutations associated with fALS were found in almost 20 additional genes, most of which were associated with only 1–5% of fALS cases, although C9ORF72, a newly identified gene of unknown function, is linked to 30% of fALS [11]. Many ALS-associated genes were identified using linkage analysis and candidate gene sequencing on DNA from affected families, although some were discovered through histological associations.

For example, the TDP-43 protein was found to be present in motor neuron inclusion bodies in the brain and spinal cord of many patients with ALS [12], suggesting gene dysregulation. Mutations in the TAR DNA-Binding Protein (TARDBP) gene, responsible for TDP-43 protein synthesis, were later identified by gene sequencing of patient DNA [13]. The wild-type form of TDP-43 interacts with another ALS-associated protein, FUS, both of which can bind RNA and DNA, and are involved in transcriptional repression, pre-mRNA splicing, and translation regulation [14,15]. However, the function of FUS and TARDBP in patients with ALS carrying the mutated form of the genes remains unknown.

Soon after the discovery of ALS-associated genes, animal models expressing mutant human ALS-associated genes were developed and widely adopted to study the mechanism of action behind variants in vivo [16]. Given that sALS and fALS are clinically similar and believed to have similar pathological mechanisms [17], mouse models of fALS were thought to represent general ALS pathology in terms of identification of interventions to slow or reverse the disease progression. Over the past 20 years, although rodents, especially mice, have been the most commonly used models for ALS [18], different species, including Caenorhabditis elegans [19], Drosophila [20], zebrafish [21,22], and even nonhuman primates [23], have also been used as model organisms. Here, we describe the most common nonhuman animal models of ALS and discuss the caveats influencing their validity and translational applicability (Table 1). Additionally, we summarize current, and suggest potential additional, alternative methods for studying ALS progression and identifying therapies, with a focus on in vitro and non-animal approaches to improve translational efficiency and benefit the patients.

Current use of nonhuman animals

Various species, from vertebrates to invertebrates, have been used to develop ALS models. We have been able to learn about ALS from those models, but, as with any model system, there are limitations in their ability to recapitulate the disease and in the knowledge gathered from their use. The ‘ideal’ model would reproduce the human disease symptoms identically and with the same progression (‘face’ validity) and neurobiological mechanism of action (construct validity), while also serving as a platform for the evaluation of therapeutic interventions in humans (predictive validity).

Canine degenerative myelopathy

No species other than humans are known to naturally develop ALS; however, dogs can experience a similar neuromuscular disease with some ALS-like clinical features. Currently, two SOD1 mutations, E40K and T185S, have been identified by genome-wide association and sequencing studies with canine degenerative myelopathy (DM) [24,25]. DM and ALS are similar in that both are age-related, fatal diseases with progressive loss of both upper and lower motor neurons with subsequent muscle degeneration. The diseases are also similar histologically: spinal cords of dogs with DM and humans with ALS exhibit lesions, ubiquitin-positive inclusion bodies, signs of oxidative stress, and neuromuscular denervation [24,26]. However, there are significant differences between the two diseases. Canine DM begins with upper motor neuron defects [24], whereas human ALS dysfunction begins in either upper or lower motor
neurons [27]. Most human SOD1 mutations are dominant [27], whereas DM appears to be recessive with incomplete penetrance [24], suggesting that SOD1 functions or requirements are unique in humans. Additionally, men are more susceptible to ALS than women and most ALS is sporadic [3,28], whereas DM in dogs is equal between the sexes, although there is breed susceptibility, and generally occurs in a familial pattern [29].

The similarities to ALS indicate that information regarding the mechanism and progression of DM may yield insight into ALS. Most histological research on ALS is done on tissue from individuals who died at late stages of disease progression and little is known about the manifestations of early-stage ALS. However, dogs with DM are often euthanized early in disease progression because of deterioration in their quality of life [24] and, therefore, early-stage DM disease tissues are available.

## Transgenic animals

Rodents have been preferred to other species because of their more complex central nervous system (CNS), and because they are easier to handle and have a short time to manifest disease-like phenotypes. Nonhuman primate models have also been developed based on their presumed relevance because of phylogenetic similarity with humans, but have significant disadvantages, including time, space, funding, and ethical concerns [23].

The zebrafish is a unique organism in that it has a conserved, simplified vertebrate nervous system, a short lifespan, and is amenable to genetic manipulation and therapeutic screening. Furthermore, 70% of human genes have at least one zebrafish ortholog, and a recent study showed that 82% of genes associated with human diseases have a zebrafish ortholog. Researchers studying ALS have found that the use of zebrafish models provides unique insights into systemic, cellular, and molecular pathways associated with different genetic variants. The flexibility of this model and similarity to the human genome have influenced its rising popularity in neurodegenerative research and has increased our understanding of the biological activity of genes associated with human diseases. However, despite evolutionary relations or anatomical similarities between zebrafish and human, biochemical mechanisms or physiological responses are different, and data accumulated using zebrafish are often not relevant to humans. Invertebrate models, such as C. elegans and Drosophila, have also been used to describe the disease phenotype and progression at the cellular and molecular levels in a relatively cell-autonomous manner, despite their lack of lower motor neurons.

### Table 1: A summary of ALS research methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>Examples</th>
<th>Features</th>
<th>Limitations</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td><em>Caenorhabditis elegans</em></td>
<td>Cellular focus without systemic complexity</td>
<td>Lacks spinal cord and lower motor neurons affected by ALS</td>
<td>[19,30,43]</td>
</tr>
<tr>
<td></td>
<td><em>Drosophila</em></td>
<td>More complexity than <em>C. elegans</em>, but less than vertebrates</td>
<td>Wild-type human gene expression is toxic; relatively simplistic compared with human systems</td>
<td>[20,35,44]</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Many different models; short lifespan and disease length</td>
<td>Not mammalian; relatively new models</td>
<td>[21,31,36]</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Larger size; short lifespan and disease length</td>
<td>Overexpression artifacts; unique biological properties from humans (lifespan, metabolism, etc.)</td>
<td>[18,37,38,80]</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>Naturally occurring disease (DM)</td>
<td>Disease phenotype differences from mouse DM similar to, but not same as ALS</td>
<td>[22,46,80,107]</td>
</tr>
<tr>
<td></td>
<td>Nonhuman primates</td>
<td>Better disease mimic than other species</td>
<td>Expensive long lived, ethical issues</td>
<td>[24,26,29]</td>
</tr>
<tr>
<td>In vitro cultures</td>
<td>Yeast</td>
<td>Simple, models protein aggregation well</td>
<td>Not systemic, neural, or even human</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>Patient dissociated motor neurons</td>
<td>Natural disease gene expression; easy, direct drug application</td>
<td>Finite lifespan; difficult to transfect</td>
<td>[36,86,87]</td>
</tr>
<tr>
<td></td>
<td>NSC34</td>
<td>Immortal motor neuron-like line; human; manipulable</td>
<td>Induced disease phenotype; not true motor neuron line</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>Stem cells</td>
<td>Can transplant or differentiate for study/transplant</td>
<td>Not systemic, transplantation still experimental</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>iPSCs</td>
<td>Easily manipulated; more systemic than cell cultures</td>
<td>Same recapitulation issues as whole animal; distant connections severed</td>
<td>[23]</td>
</tr>
<tr>
<td>In silico</td>
<td>Computer modeling virtual mice</td>
<td>High throughput, hypothesis driven</td>
<td>Requires <em>a priori</em> knowledge; underdeveloped</td>
<td>[23]</td>
</tr>
<tr>
<td>Genetic</td>
<td>NGS</td>
<td>Identifies sequence mutations</td>
<td>Requires previous linkage; requires several affected individuals with same mutation</td>
<td>[11,82,83]</td>
</tr>
<tr>
<td></td>
<td>GWAS</td>
<td>Identifies SNPs linked to disease; can identify several SNPs at once</td>
<td>Need large sample size; genetic heterogeneity in ALS population; remaining mutations can be risk related</td>
<td>[11,82,83]</td>
</tr>
<tr>
<td></td>
<td>Molecular phenotyping</td>
<td>Identifies clinical targets; gives insights into disease pathogenesis</td>
<td>Expensive at high throughput levels; direct versus indirect targets of disease unclear</td>
<td>[11,82,83]</td>
</tr>
<tr>
<td>Available data</td>
<td>Patient data</td>
<td>Cheap, quick, easily accessible; large sample sizes</td>
<td>Relies on personal reporting; limited data set breadth</td>
<td>[84,85]</td>
</tr>
<tr>
<td></td>
<td>Medical records</td>
<td>Easy trend analysis; less subjective than personal reporting</td>
<td>Limited data set breadth</td>
<td>[84,85]</td>
</tr>
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</table>
SOD1 transgenic animals. Since the discovery of SOD1 as an ALS-associated gene in 1994, a variety of transgenic SOD1 mutant animal models have been created. More than 150 mutations of SOD1 have been identified in fALS and over 20 rodent models have been generated, most of which overexpress a mutant variant of human SOD1. The oldest and most widely use model is the G93A hSOD1 overexpression mouse, which at one point was used in up to 97% of ALS drug studies [18]. Transgenic mutant human (h)SOD1 overexpression models also exist in C. elegans (G93A, G85R, G37R, and A4V) [30], Drosophila (G85R and A4V) [20], and zebrafish (G93R, G37R, and A4V) [21,31].

The SOD1 models were initially developed to be a general model for ALS (SOD1-related and non-SOD1-related fALS, as well as sALS). Although SOD1 mutations are not present in sALS, there are phenotypic similarities with fALS, and it has been assumed that familial and sporadic forms shared the same neuronal degeneration pathway and studying the familial form could provide insight into the sporadic form. However, the validity of these assumptions has been questioned. It has been shown that tissues from patients with sALS and non-SOD1 fALS exhibit aggregates of TDP-43 in motor neurons and glia [32], yet patients with ALS and SOD1 mutations display TDP-43-negative neural aggregates [32]. The G93A hSOD1 mouse mimics the human SOD1 phenotype with TDP-43-negative aggregate staining [33], indicating that mouse SOD1 models may better represent the phenotype of patients with ALS and hSOD1 mutations, but not those with sALS or non-SOD1 fALS mutations, which account for 98% of ALS cases.

TARDBP transgenic animals. In 2008, TARDBP was identified as an associated gene in 3–5% of fALS cases and 2% of SALS cases [34]. Since then, mutant human TARDBP has been overexpressed in both C. elegans and Drosophila, which exhibit some human ALS characteristics, including loss of motility and reduced lifespan [35]. The zebrafish TARDBP model is not yet well characterized, but does exhibit motor defects and axon deficiencies [36]. Mouse and rat overexpression models appear to recapitulate overarching human ALS phenotypes in that they exhibit neuronal ubiquitin-positive inclusions, motor neuron degeneration, axon degeneration, motor loss, paralysis, and death [37,38]. Given concerns about the lack of relevance of expressing models that exhibit early paralysis and death, a recent model was developed expressing lower levels of TDP-43 to mimic the human protein expression level [39]. To date, one nonhuman primate TDP-43 model has been developed by overexpressing the human wild-type from of TDP-43 in the spinal cords of cynomolgus monkey to recapitulate the redistribution of TDP-43 from the nucleus to the cytoplasm, which was not observed in rat models [23].

Mutant models of TARDBP expression are still relatively new, and lack characterization in the context of the human TARDBP ALS phenotype, but recent publications have reported that SOTARDBP mutations might be associated with ALS, whereas others report that those mutations are still rare in ALS [40,41].

FUS transgenic animals. In 2009, FUS was identified as an associated gene in 5% of fALS cases and 1% of SALS cases [42]. Inclusions of FUS in the cytoplasm have been shown to be recurrent in patients with ALS and FUS mutations. Since then, a handful of models overexpressing the mutant human FUS (hFUS) have been created in C. elegans, Drosophila, zebrafish, and rat. Both the mutant hFUS overexpression Drosophila and C. elegans models exhibit a decreased number of neuromuscular junctions, motor neuron damage, cytoplasmic FUS-positive protein aggregation, locomotor impairment, and premature death [43,44]. Overexpression of mutant hFUS in zebrafish is not fully characterized, but does result in motor neuron defects and motor deficits [45]. In rodents, it looks like the overexpression of mutated or wild-type FUS induces different phenotypes depending on the species. The mutant hFUS overexpression rat model exhibits protein aggregates, neurodegeneration, and muscle atrophy [46], while recent studies have shown that overexpression of wild-type FUS in rats induces cognitive defects in aged animals without motor phenotype or spinal cord pathology. However, in mice, overexpression of wild-type FUS induces motor neuron degeneration when significant amounts of protein accumulate in the cytoplasm [47].

Concerns regarding the relevance of nonhuman animals to ALS

Given their ability to recapitulate some key ALS features, animal models have been used to study ALS for almost two decades; however, the validity of animals as models for human diseases has been challenged and is generally debated [18,48–51] (http://dana.org/News/Details.aspx?id=42802). Often, the underlying mechanisms of action and resulting clinical manifestation found in animal models not only do not correlate with that in humans, but also guide researchers and resources along fruitless avenues. Studies have identified troubling disparities between histological phenotypes of popular ALS animal models and human ALS [32,33]. Disparities also exist within research results in the same models at the same or different institutions, indicating a lack of reproducibility [18]. Additionally, some concerns have been raised regarding the different forms of cell death that may exist between humans and mice models of ALS [52]. Although animal models have yielded significant information regarding biology, their use in modeling complex human disorders is limited.

Overexpression issues

Although most broad disease clinical characteristics, such as motor neuron degeneration, motor loss, and death, are replicated in some animal ALS models, the validity of overexpression models has been called into question. It is unknown whether ALS-associated genes are upregulated in patients with ALS, yet most animal models of ALS utilize overexpression of a mutant human ALS-associated gene. As described above, the overexpression of FUS using different promoters and onset of expression in different species (rats and mice) and strains result in different phenotypes [46,47]. Models resulting from gene overexpression have several to hundreds of copies of the human transgene and expression of the mutant human gene is several-fold higher than endogenous animal gene expression [53]. In the most popular mouse model (G93A SOD1), 25 copies of the human transgene have been inserted and hSod1 is expressed at levels 13-fold higher than endogenous mSod1 [53]. Interestingly, overexpression of wild-type genes in mice causes some neuronal defects and vacuole accumulation, which are not observed in humans [47,54], likely indicative of mitochondrial swelling from toxic protein overload. Consistent with an overload hypothesis, the exogenous gene copy numbers of either mutant or wild-type genes in mice is strongly correlated with disease phenotype severity [55,56]. Overexpression effects are also
seen in *Drosophila*, where wild-type hTARDBP overexpression causes dose-dependent motor defects and severe reduction of lifespan [57]. However, depending on the level overexpression of proteins linked to ALS, non-ALS-relevant phenotypes can be observed, ranging from photoreceptor degeneration to disruption of eye architecture and larval lethality, highlighting the toxic effects and lack of specificity of this model.

Overall, these studies suggest that gene overexpression causes toxic effects, some of which are similar to an ALS-like phenotype but are not related to ALS disease progression, thereby complicating disease recapitulation and understanding. Animal models are created to understand disease progression and pathogenesis, support the development of treatments, and collect preclinical data; artifacts of gene overexpression can confound research results, mislead future studies, and, therefore, delay the availability of new drugs to patients.

**Species biological differences impact translational potential**

As mentioned above, models of ALS in *C. elegans* and *Drosophila* are similar to human ALS in that both exhibit inclusion bodies, defects in neuromuscular junction signaling, glial abnormalities, progressive motor defects, and even death [20,30,35,43]. However, those models do not exhibit motor neuron death [20,30], as observed in humans with ALS, who lose both upper and lower motor neurons. This difference could be due to the short lifespan of these species or to the differences between the neural cell physiology of vertebrates and invertebrates. Zebrafish G93R mutants resemble ALS in that they exhibit the main disease characteristics, including spinal motor neuron loss, muscle degeneration, motor deficits, neuromuscular junction defects, paralysis, and early death [31]. However, the transgenic zebrafish model is relatively new and not yet fully understood, and developing new interventions for human ALS based on studies conducted on zebrafish is not a guarantee of success, despite its resemblance to the disease in humans.

Similar to zebrafish, some rodent models of ALS, including SOD1 models, also appear to recapitulate many of the characteristic ALS phenotypes, including neuromuscular junction disruption, upper and lower motor neuron loss, muscle degeneration, respiratory problems, immune abnormalities, blood-brain barrier disruptions, and protein aggregation [16,58–60]. Transgenic models, such as SOD1 and TARDBP models, also demonstrate progressive motor decline that eventually leads to paralysis and death. However, some strains of G93A SOD1 transgenic rats have more aggressive disease phenotypes and different motor neuron degeneration patterns compared with their mouse counterparts [22], indicating unique disease manifestations among species. Given that transgenic animals, such as G93A SOD1 rodents exhibiting some similarities with ALS, display species-specific disease phenotypes, this suggests that effects observed in animal models cannot fully be translated into humans because of critical biological differences.

These biological differences can influence disease manifestations, treatment responses, and toxicological thresholds. For example, administration of certain chemicals, such as methyl tert-butyl ether (MTBE), a fuel additive, in male rats induced endogenous α2u-globulin nephropathy and led to kidney tumors; however, humans do not produce α2u-globulin and, therefore, respond differently [61,62]. Similarly, penicillin is safe in humans, but toxic to guinea pigs [63]. In other instances, drugs are harmful to humans but not animals, as is the case with thalidomide. Exposure of pregnant women to thalidomide during the 1970s resulted in severe fetal developmental defects; however, thalidomide testing in animals had shown inconsistent and species- and/or strain-specific responses [64,65]. Species variations in drug toxicology responses are likely due to differences in the uptake and/or excretion, metabolism, and distribution of drugs, as well as immune responses [66,67]. These critical differences indicate that animals and animal models may not be ideal candidates for the toxicology testing that is necessary for establishing the safety of new drugs.

**Aging, environment, gender, and CNS physiology**

ALS is strongly age associated, but the lack of age-related ALS-like diseases outside of humans (with the possible exception of dogs) suggests the need to appreciate the biological differences between humans and other species. Each species has a unique lifespan, tissue-specific cellular turnover rates, oxidative stress responses, and gene-expression profiles, all of which contribute to aging, and are likely to contribute to differences in aging-related disease progression.

Aging and ALS are also linked to local factors, such as environment, diet, and stress [68,69]; however, different animal-housing facilities and research groups have varying animal colony management methods, which affect animal stress and behavior [70–72]. The same animal model studied at two different facilities or by two different investigators can exhibit different disease features, thus confounding results throughout the field. Additionally, gender is a risk factor for ALS, because men are slightly more likely to develop the disease than women and female ALS-model mice tend to live longer than males [28,73]; however, gender is not always taken into account in animal studies [18], which can contribute to poor reproducibility. Additionally, although the human genome is approximately 97.5% similar to the mouse genome, differences in DNA methylation or histone modification can be very different among species, leading to variability in gene expression.

The anatomy and physiology of the organ systems affected by ALS (CNS and skeletal muscle) also differ between species, which likely influences differences in disease phenotype and drug responses. Important anatomic brain differences exist between species [74,75], and even between strains within a single species [76]. According to Eisen, ALS and other neurodegenerative diseases are human-specific diseases. Indeed, the evolution of *Homo sapiens* led to neocortical changes, with the development of new cortical areas and increased interconnections that are not only responsible for the acquisition of bipedalism and opposable thumbs, but also the brain regions targeted by ALS [77]. Spinal cord anatomy between species is also unique; the length of the human spinal cord is several times the size of the cord in most animals. Species also differ at the molecular level, because rodents and humans exhibit unique gene expression patterns in the brain [78]. These differences in physiology, complexity, and expression undoubtedly affect the ability of animal models to mimic human neural disease phenotypes.

**Concerns about the methodologies used to create animal models of ALS**

Given that transgenic animals are developed using inbred strains that are relatively genetically homogenous, results using the same
transgenic line should be reproducible by different research groups. However, this has often not been the case for ALS animal models for several reasons. As mentioned above, some differences result from animal colony management protocols. In these models, disease progression is often assessed based on qualitative measures, such as grip strength, wire hanging, gait analysis, and neurological scoring [79]. The lack of standard quantitative measures makes it difficult to compare results between studies. In addition, drug studies on transgenic mice are often underpowered, lack randomization of groups, and outcome evaluations are not performed blind [18,80]. In fact, the lack of standardized methodology led the European ALS/MND group to develop and publish the Standard Operating Procedures for ALS preclinical animal research [79]. However, it is still too early to tell whether these guidelines will be used or impact the reproducibility of results.

**ALS genetic subpopulations can have unique disease progression mechanisms**

As mentioned above, transgenic animals, especially rodents, are established in inbred and, therefore, genetically homogeneous lines [18]. However, patients with ALS are clinically and genetically diverse. As a result, drug studies in ALS mouse models showing small but positive effects have not translated to positive findings in human clinical trials, likely because the effects of the drugs are both small and specific to unique rodent genetic backgrounds.

According to the guidelines for preclinical testing and colony management when working with ALS mice, established by The Jackson Laboratory and Prize4Life, mixed strains of ALS animal models have an advantage because they better mimic the heterogeneous human population. However, mixing strains between experimental groups could require an increased number of animals to reach statistical significance from a heterogeneous subject pool. The use of inadequate numbers of animals can negatively impact reproducibility and result in misleading data and wasted resources. Genetic issues can even occur within a single strain; G93A hSOD1 mouse colonies can spontaneously lose copies of G93A hSOD1, resulting in misleading data from delayed disease onset and death [56]. The variable genetics of ALS animal model colonies is a complicating factor that can easily affect the accuracy and reproducibility of results, and likely has contributed to the confusion within the ALS field.

**Clinical trial design**

In most studies, potential drugs or compounds are tested on animal models before the onset of symptoms, which is not relevant when it comes to clinical trials because it is impossible to start treating patients presymptomatically. Additionally, Vin sant et al. proposed that the disease onset in SOD1 mutant mice starts 2 months before it was initially thought (postnatal day 30 instead of 90); therefore, the author suggested that treatment should be tested at postnatal day 30 [81]. According to the Guidelines for preclinical testing and colony management, it is recommended to conduct preclinical trials pre-onset and, if the drugs show some effect, to conduct the experiments at or post-onset; therefore, there is no valid reason to conduct the experiments pre-onset on animals if they will eventually be conducted at or post-onset.

**Human studies and non-animal approaches**

### Genetic studies in humans

Previously, genetic studies have been instrumental in identifying associated mutations in ALS. The identification of C9ORF72 through next-generation sequencing (NGS), which had previously been linked to fALS using a genome-wide association study (GWAS) [11,82], was a huge breakthrough in the ALS field, because C9ORF72 is an associated mutation responsible for up to 24% of fALS and 4% sALS cases [82]. Given that the genetic causes of most sALS cases are still unknown, further GWAS and NGS studies would be useful to identify other ALS-associated genes. Furthermore, genetic studies can improve our understanding of ALS progression and mechanisms. Molecular phenotyping studies have identified unique gene expression profiles of spinal cord neuronal populations in controls versus patients with ALS [83], implicating certain pathways in ALS progression. Future molecular phenotyping studies could identify early versus late markers, thereby potentially informing causal relations.

### Human data and population monitoring

For years, researchers have combed available patient data for causative links to ALS from several patient registries, including ALS registries [National ALS Registry, Department of Veteran’s Affairs (VA) ALS Registry, and fALS Connect], which contain data from thousands of patients as well as medical records. These databases are easily and quickly accessible, free, contain data on a large patient population, and, therefore, have benefitted researchers. Registry data can be used to identify genetic and environmental ALS risk factors, predict disease progression, or even improve treatment [84,85].

Additionally, monitoring populations susceptible to the disease could help identify biological or physical markers, which would give us a better understanding of the progression of the disease and, therefore, identify early diagnostics.

### Cell and tissue models

Yeasts have been used to study molecular interactions associated with neurodegenerative diseases, such as ALS. Yeasts are amenable to genetic manipulation, grow quickly, are easy to study at a single-cell level, and have many of the basic cellular mechanisms involved in neurodegeneration, such as mitochondrial defects, protein misfolding, and protein trafficking/degradation impairments. As such, yeast can be used to study cellular responses to known ALS genetic mutations to determine the underlying mechanisms of ALS disease pathology. For example, yeast studies showed that TDP-43 is normally localized to the nucleus, but TDP-43 overexpression resulted in cytoplasmic, toxic protein aggregates [86]. Other ALS mutations increased cellular stress and also resulted in aggregates [87]. Of course, ALS is a complex, multicellular disease and, thus, as a single-celled organism, yeast is not capable of recapitulating ALS in its systemic entirety. It is also impossible with yeast to interrogate the complex interactions with other proteins or pathways solely in human cells or in the particular cell of interest, the motor neuron. However, yeast as a model has proven useful in delineating the ALS disease pathway at the molecular and cellular levels, which is a critical foundation upon which to build understanding of the disease at the tissue and multiorgan levels.
Other cultured cells are similarly useful in delineating the fundamental molecular disease pathway. Cultures can comprise primary animal and/or human cells from any tissue, immortal nonstem cell lines, and even embryonic, fetal, or adult stem cells. The recent discovery of induced pluripotent stem cells (iPSC) holds great promise because they can be generated from those affected by ALS, maintaining the donor genotype, and provide an unlimited source of cells for modeling the disease. In case of primary animal cells, neural subtypes from different species can be used, thereby improving species and cellular relevance for both pathogenesis studies and drug screenings [88]. For example, motor neuron and motor neuron-like cultures (such as NSC34 cells) display cell stress, aggregation, and cell death in response to ALS mutations [36,87]. Additionally, cell cultures can generate cell populations for transplantation therapies; everything from embryonic stem cells to adult neuroblasts can be left undifferentiated, induced to differentiate, or altered and then implanted [89].

Cell culture models are even beginning to supplement, and at times replace, animal testing for toxicological studies because they are quicker, cheaper, more effective, and more ethical [90].

Similar to yeast, cell cultures are limited with respect to understanding systemic functions and defects; however, culture models are useful for identifying cell-specific defects and mechanisms. While groups, such as the organ-on-a-chip program funded by DARPA, NIH, and FDA, are beginning to develop complex engineering platforms capable of modeling interactions between multiple organ systems, they are still immature and have not shown much utility (http://www.ncats.nih.gov/research/reengineering/tissue-chip/tissue-chip.html). However, in the future, it is possible that these platforms will enable cell culture systems to better model complex in vivo environments.

Tissue slice cultures add complexity and, therefore, relevance relative to single cell-type cultures. Cultured tissues maintain local environments and connectivity, but allow relatively easy access to molecular and environmental manipulations. As such, slice cultures are ideal for understanding cellular and local system pathways underlying ALS disease progression and mechanisms. Although they still require animals as a source of the tissue, experiments with cultured tissues use fewer animals per experiment, allow multiple testing per animal, and are also quicker, cheaper, and more amenable to treatments targeting gene expression. For example, tissue slice cultures can be transplanted using viruses or particle-mediated delivery [91,92], which can be used to either increase or knockdown target gene expression. Furthermore, this technique has recently shown promising data in SOD1 mice models [93]. Unfortunately, slice cultures also have several disadvantages. They require living tissues, which are not widely available from humans. ALS mouse model tissues have questionable validity, as previously noted for transgenic animals. Additionally, ALS is an age-related disease, but slice cultures are not recommended for tissues from older animals because of poor survival responses to mechanical trauma induced during the preparation.

For all of these tissue culture approaches, drug-screening studies would strongly benefit from the identification of cellular markers of disease progression and health. Marker levels could be used to quickly determine treatment efficacy and even toxicity in a species-specific manner. This would also provide an important qualitative and quantitative method to compare the response of tissues from different ALS genotypes. Additionally, use of markers could help standardize results between laboratories.

In vitro models using iPSCs

iPSCs represent the opportunity to integrate the complex genomic landscape seen in humans affected by both fALS and sALS. Human iPSCs are derived from adult somatic cells through epigenetic modification to yield pluripotent cells that proliferate indefinitely in the undifferentiated state, yet retain the ability to differentiate into organ-specific cell types. Motor neurons and other relevant cell types, such as astrocytes, are recreated from iPSCs obtained from humans with ALS and healthy controls can be generated to evaluate genetic factors related to the disease as well as exposure to various toxins. The differentiated cells are characterized and compared in the hope of identifying cellular phenotypes that recapitulate key aspects of the disease.

Although these cells are not free of caveats, such as the difficulty to accurately define the state of the cells, mature the cells, or obtain the right cells, they hold great promise for ALS research, but effective integration is contingent upon access to large numbers of affected cell lines and reproducible panels of their differentiated progeny. The first study demonstrating the ability to generate iPSCs from an 82-year-old woman with ALS and differentiate them into motor neurons was published in 2008 [94]. More recent studies have evaluated various cellular phenotypes related to the progression of ALS [95,96]. If cellular phenotypes are successfully identified, the iPSC-based model can then be used to screen drugs that correct the phenotype, thereby generating a model of human efficacy using human cells. Recently, researchers conducted a screen of small molecules on mouse embryonic stem cells differentiated into motor neurons and identified one molecule, kenpaullone, which was found to prolong the survival of both wild-type and SOD1 mutant motor neurons. The authors also found, by testing kenpaullone on human motoneurons derived from human embryonic stem cells or iPSCs, that the compound showed promising preclinical results. Their study also demonstrated that dexamphetamine and olmesartan, two compounds that are active in ALS mouse models and that failed in Phase III clinical trials, had no or too small an effect on human-derived motor neurons [97]. In a more recent study, researchers were able to recapitulate a clinical phenotype, specifically increased excitability of motor neurons, measured from patients using iPSC-derived motor neurons in a dish. Through the screening of drugs known to reduce neuronal excitability previously approved for treatment of epilepsy, they showed that one drug, retigabine, reversed the cellular phenotype [98]. This work is now the basis for a clinical trial supported by Glaxo-Smith Kline and represents the first drug identified using iPSC-derived tissue as a disease model.

In silico models

As computer modeling becomes more sophisticated, software can be used to make predictions, which could influence the direction of in vivo and in vitro ALS studies. In silico modeling programs can be used to make a variety of predictions, which can save time, money, and lives of both humans and animals. For example, software can gauge the functional variability between normal and mutant proteins and can suggest whether mutations result
in a gain or loss of function. Most known ALS gene mutations result in proteins with a toxic gain in function; however, recent software predictions indicated that mutations in the novel ALS gene, SQSTM1, result in a loss of function [99]. Software programs also predicted mechanisms of disease development in SOD1 mutants [100,101]. Additionally, virtual mice are being created from well-characterized mouse models [102,103], and can be tested for drug efficacy and, perhaps more importantly, studied for species-specific differences in disease manifestation [102,104]. These prediction programs will be critical to understanding disease pathogenesis and targets for treatment, both in the general ALS population and in individuals with unique mutations. However, prediction software requires a priori knowledge of associative genes and their mutations.

**Predictive outcome pathways**

In the chemical field, the Organization for Economic Cooperation and Development (OECD), the US Environmental Protection Agency, and the European Union Joint Research Centre are collaborating to develop the necessary infrastructure to host a unified ‘knowledge base’ of adverse outcome pathways (AOPs) that covers the broad spectrum of biological pathways that are likely to be involved in human health and ecological risk assessment: the Adverse Outcome Pathway Knowledge Base (AOP KB) (http://ihcp.jrc.ec.europa.eu/our_activities/alt-animal-testing-safety-assessment-chemicals/improved_safety_assessment_chemicals/first-release-of-aop-wiki; http://www.epa.gov/research/priorities/docs/aop-wiki.pdf; http://aopkb.org/aopwiki/index.php/Main_Page; http://www.epa.gov/nctc/download_files/chemical_prioritization/AOPWikiTutorial%20v2.pdf). Building on the existing AOP KB, we could develop a new pathway knowledge base for different for disease models to understand the disease and identify new therapeutics targets in a more efficient manner.

ALS is a complex disease with a spectrum of phenotypes that could represent several closely related disorders with different causes but similar phenotypes. Predictive outcome of disease models would benefit from the use of disease pathway approaches, similar to AOPs used in toxicology [105]. Scientists and clinicians have already gathered much information on ALS pathways, some of which are integrated in a KEGG pathway map (http://www.genome.jp/dbget-bin/www_bget?pathway:map05014). However, we need to focus on integrating this information in a common and accessible database where sequences of molecular changes within the cells leading to the development of the clinical conditions could be listed, as described in Fig. 1. This approach would allow for new information to be added as it is discovered to get a broader understanding of a disease such as ALS, or of specific populations, and to identify new therapeutic targets [106].


**FIGURE 1**

Adverse outcome pathway. (a) The adverse outcome pathway is a biological map from the molecular initiating event through to the resulting adverse outcome that encompasses both mechanism and mode of action. (b) Adaptation of the pathway-based approach to the amyotrophic lateral sclerosis (ALS) models describing the key events leading to the adverse outcome at the organism level. Adapted from [105] (a).
Similarly, it would be possible to map and understand the disease process of each of the different forms of ALS by using computer technology, genetic, and epidemiology databases. This would enable scientists to develop targeted and effective management drugs and cures. Lessons can also be learned from data generated during clinical trials. Understanding why a clinical trial failed is in the best interest of patients and represents an important piece of the disease pathways map.

**Concluding remarks**

The lack of clinical translation from animals to humans after 30 years of research suggests that animal models are not an ideal system for studying ALS or for developing drug therapies. The disease mechanism data from animal model studies are not cohesive and the preclinical drug studies have not translated well into successful human clinical trials. Given that animals do not naturally develop ALS, the current models are animals subjected to artificial gene expression and disease construction. However, these constructed models have phenotypes distinct from human ALS, thereby limiting model validity and muddying the analyses of an already complex disease. In addition, the relevance of animals as models in general is being questioned. Critical biological differences exist between animals and humans, resulting in unique disease phenotypes, therapy responses, and toxicity thresholds. Advances in molecular biology, human disease biotechnology, and computer science could be leveraged to drive investments in developing new, potentially more relevant and effective research methods, including *in silico*, disease pathway approach- and human-based studies. Redirecting funding toward these models would considerably improve their efficacy, validity, and ubiquity, as well as adding to our understanding of human biology, disease progression, and drug functions. Importantly, using a variety of alternatives will improve data validity and deepen our understanding of basic science. Alternative techniques are not only ethically superior, but also offer more direct, robust insights into human biology and disease, thereby accelerating the transition from bench to bedside. Given that the US Congress is re-examining the way in which drugs are discovered, developed, and delivered through the 21st Century Cures Act, and as the ALS association has received more than US$100 million through the ‘Ice Bucket Challenge’, a large investment is being made directly to help the families of patients with ALS. The time is ripe for re-examining our approach to ALS intervention; it is clear that we need to adopt a new approach to biomedical research based on human biology and the use of new and advanced technologies.

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