Review

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Modeling ALS using iPSCs: Is it possible to reproduce the phenotypic variations observed in patients *in vitro*?

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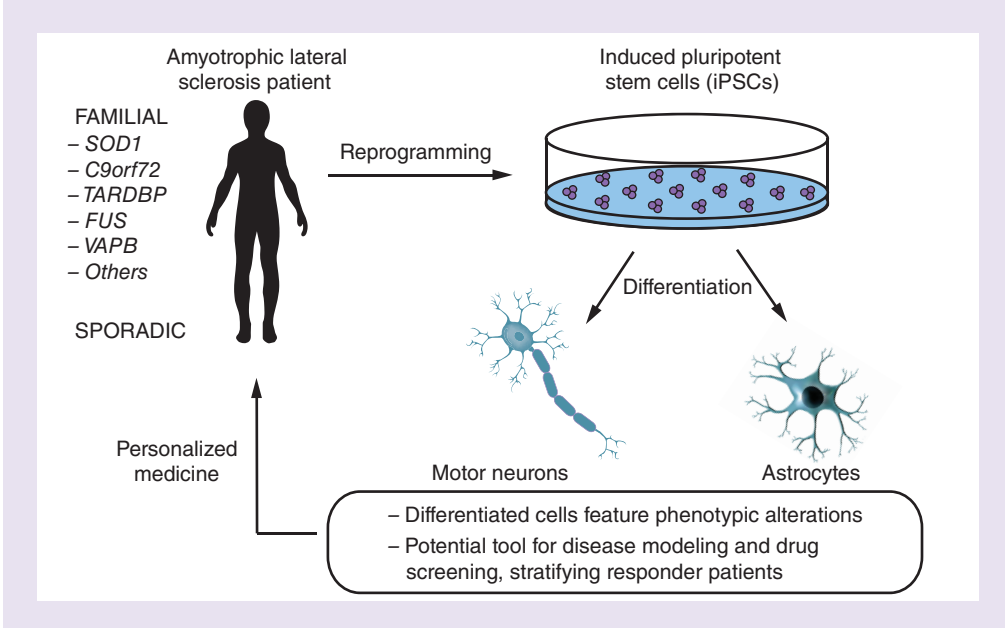
Amyotrophic lateral sclerosis (ALS) is a fatal disease that leads to progressive degeneration of motoneurons. Mutations in the *C9ORF72*, *SOD1*, *TARDBP* and *FUS* genes, among others, have been associated with ALS. Although motoneuron degeneration is the common outcome of ALS, different pathological mechanisms seem to be involved in this process, depending on the genotypic background of the patient. The advent of induced pluripotent stem cell (iPSC) technology enabled the development of patient-specific cell lines, from which it is possible to generate different cell types and search for phenotypic alterations. In this review, we summarize the pathophysiological markers detected in cells differentiated from iPSCs of ALS patients. In a translational perspective, iPSCs from ALS patients could be useful for drug screening, through stratifying patients according to their genetic background.

Graphical abstract:



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Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disease described almost 150 years ago by the French physician Jean-Martin Charcot. Its hallmarks are muscle atrophy and progressive

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degeneration of superior and inferior motoneurons (MN) and their axons in the lateral spinal cord and peripheral nerves [1]. ALS is the most common MN disorder in adults and eventually leads to mortality, usually due to paralysis of the respiratory muscles, on average 3–5 years after disease onset [2]. With no effective treatment available to date, the only two drugs approved by the US FDA for use in ALS are riluzole and edaravone, both of which extend life expectancy by only a few months and in a limited number of patients [3].

About 10% of ALS patients have at least one relative stricken by the disease, and these cases are classified as the familial form of ALS (fALS). When no other individual in the same family is affected at the time of diagnosis, the patient is said to have the sporadic form of the disease (sALS), which comprises approximately 90% of cases. Over a hundred mutations in different genes have been associated with fALS, increasing susceptibility to the disease and/or altering its clinical progression [4]. Variants of the genes *SOD1*, *C9orf72*, *TARDBP* and *FUS* are the most frequent disease-causing genetic alterations in familial cases [5]. Although mutations in some of these genes have also been observed in sALS patients, genetic risk factors in the sporadic form of ALS remain incompletely understood [6]. The first ALS-related mutation described was in the *SOD1* gene, in 1993 [7]. *SOD1* mutations account for about 15 and 30% of fALS in Europe and Asia, respectively. About 1.5% of sALS patients on both continents also show genetic alterations in *SOD1*. However, the most prevalent genetic modification in American and European fALS, present in more than 30% of cases, is an expansion of a hexanucleotide intronic repeat (GGGGCC) in the first intron of the *C9orf72* gene. *C9orf72* expansions are also directly related to frontotemporal dementia (FTD), which explains the clinical overlap between ALS and FTD. Mutations in the *TARDBP* and *FUS* genes are present in about 4 and 3% of European fALS cases, respectively [8]. *TARDBP* codifies TDP-43, the main protein constituent of cytosolic inclusions found in sALS [9]. The FUS protein shares this important physiological feature with TDP-43: mutated FUS tends to mislocalize in the cytoplasm, generating protein aggregates. In the Brazilian population, mutations in the *VAPB* gene, especially the well-characterized P56S, are among the most frequent in fALS patients [10] and are related to a slowly progressive form of ALS, termed ALS8 [11].

Only 1 year after the identification of the first ALS-related mutation in the *SOD1* gene, the first animal model of the disease was developed: the SOD1-G93A transgenic mouse, which remains the most studied to date [12]. Although this model has provided useful insights into ALS pathology, accumulating evidence suggests the existence of several important pathological differences between SOD1-ALS and other types of ALS, especially sALS [13]. More recently, several other animal models have been developed, with mutations in *TARDBP*, *FUS*, and *C9orf72*, among other genes [14]. However, these transgenic models also have the same important limitation: they represent the pathophysiology of only a small fraction of total ALS patients, which explains the continual failure in clinical trials of drugs that performed well in pre-clinical studies. Therefore, representative models of ALS with undefined genetic backgrounds, such as sporadic cases, are sorely needed.

Almost 15 years ago, induced pluripotent stem cells (iPSC) appeared as a promising tool for *in vitro* disease modeling. In 2006, Takahashi and Yamanaka established a breakthrough protocol based on retroviral transfections of four transcription factors (Oct 3/4, Sox2, Klf4 and c-Myc) that allowed adult mouse fibroblasts to be converted into undifferentiated pluripotent stem cells [15]. In the following year, the same protocol also proved be efficient in reprogramming human somatic cells [16]. In 2008, the first iPSC derived from an ALS patient was described by Dimos and collaborators [17]. Several iPSC lines have now been derived from ALS patients with diverse genotypes.

An important advantage of using iPSCs for studying ALS is that these cells retain the donor's genetic information, which is extremely useful for sALS modeling, where no animal models are available. In addition, iPSCs can be differentiated into virtually any cell type, including astrocytes and MNs, the main target in ALS pathophysiology. Platforms based on cells differentiated from iPSCs derived from patients are a promising option for screening new drugs, allowing genetic differentiation between responder and nonresponder patients. Importantly, the cells differentiated from iPSCs must recapitulate the phenotypic alterations present in donor patients. In 2016, Park and colleagues demonstrated that MNs differentiated from SOD1-G93A transgenic-mouse iPSCs feature important hallmarks of mouse MN pathology, such as SOD1 aggregates and reduced cell viability, in comparison with MNs differentiated from wild-type mouse iPSCs [18]. In this review, we summarize the main phenotypic alterations identified in cells (astrocytes and MNs) differentiated from iPSCs of ALS patients with different genetic backgrounds (Tables 1–3). Although MNs and astrocytes are the main target cells in the study of ALS, other cell types affected by this disease might also be differentiated from iPSCs, such as oligodendrocytes [19] and Schwann cells [20], thus providing useful insights into the role of myelinating cells in ALS pathophysiology.

Table 1. Major phenotypic alterations detected in motoneurons differentiated from induced pluripotent stem cells of amyotrophic lateral sclerosis patients.

Study (year)	iPSC line genotypes	Physiological alterations detected in motoneurons *in vitro*	Ref.
Chen *et al.* (2014)	*SOD1* (D90A and A4V)	Cellular degeneration; reduced SOD1 protein levels; small SOD1 aggregates in cytoplasm, nuclei and neurites; NF inclusions; neurite degeneration.	[21]
Kiskinis *et al.* (2014)	*SOD1* (A4V)	Cellular degeneration; reduced soma size, reduction in neurite growth and number; alterations in mitochondrial morphology and motility; activation of ER stress and UPR pathways.	[22]
Wainger *et al.* (2014)	*SOD1* (A4V); *C9Orf72* expansion; *FUS* (H517Q and M511Nfs*6)	Membrane hyperexcitability.	[23]
Naujock *et al.* (2016)	*FUS* (R521C, R521L, R495QfsX527); *SOD1* (D90A, R115G); sALS	Hypoexcitability; impaired spontaneous activity; reduced Na^+ channel expression levels; elevated K^+ channel levels (except for *SOD1*-lines).	[24]
Imamura *et al.* (2017)	*SOD1* (L144FVX)	Accumulations of misfolded SOD1 protein; increased autophagy.	[25]
Wang *et al.* (2017)	*SOD1* (A272C)	Altered transcriptome.	[26]
Seminary *et al.* (2018)	*SOD1* (N139K)	Increased levels of insoluble SOD1 protein; impaired activation of HSR to increased protein burden.	[27]
	C9orf72 expansion	Increased levels of insoluble SOD1 protein; decreased number of stress granules per neuron.	
	TARDBP (M337V)	Increased insoluble TDP-43 protein.	
Bursch *et al.* (2019)	*C9orf72* expansion	Increased ChAT expression; detectable spontaneous transient electrical signals; elevated expression of glutamate kainate receptors and voltage-gated Ca^{2+} channels.	[28]
	FUS (heterozygous R521C)	Increased levels of ionotropic glutamate AMPA, NMDA and kainate receptors.	
	SOD1 (D90A)	Increased expression of metabotropic glutamate receptors.	
	TARDBP mutation	Elevated basal intracellular Ca^{2+} levels and AMPA receptor-induced signal amplitudes.	
Sareen *et al.* (2013)	*C9Orf72* expansion	Intranuclear GGGGCC-containing RNA foci; membrane hypoexcitability.	[29]
Devlin *et al.* (2015)	*TARDBP* (M337V); *C9orf72* expansion	Short-term membrane hyperexcitability; progressive loss of action-potential output; perturbations in Na^+ and K^+ currents; reduced synaptic activity.	[30]
Dafinca *et al.* (2016)	*C9orf72* expansion	Decreased cell survival; dysfunction in Ca^{2+} homeostasis; reduced levels of antiapoptotic protein Bcl-2; increased ER stress; reduced mitochondrial-membrane potential; altered mitochondrial morphology; abnormal protein aggregation and stress-granule formation; RNA foci and dipeptide repeat proteins.	[31]
Lopez-Gonzalez *et al.* (2016)	*C9orf72* expansion	Nuclear RNA foci; production of RAN translation products; increased DNA damage and p53 activation; increased ROS production and mitochondrial-membrane potential.	[32]
Selvaraj *et al.* (2018)	*C9orf72* expansion	Increased expression of glutamate GluA1 AMPA receptor subunit; increased expression of Ca^{2+}-permeable AMPA receptor; increased susceptibility to excitotoxicity; RNA foci.	[33]
Shi *et al.* (2018)	*C9orf72* expansion (ALS/FTD)	Reduced levels of C9orf72 protein; reduced vesicle trafficking and lysosomal biogenesis; accumulation of ionotropic glutamate receptors in neurite membranes; increased vulnerability to excitotoxicity; impaired clearance of neurotoxic dipeptide repeat proteins; neurodegeneration.	[34]
Seminary *et al.* (2020)	*C9Orf72* expansion	Higher levels of insoluble TDP-43 protein; autophagy impairment after stress.	[35]
Zhao *et al.* (2020)	*C9orf72* expansion	MNs co-cultured with mutant astrocytes had a more-depolarized resting membrane potential, reduction in voltage-activated currents and hypoexcitability; alterations in multiple gene pathways.	[36]
Bilican *et al.* (2012)	*TARDBP* (M337V)	Elevated levels of TDP-43 protein; reduced cell viability.	[37]
Egawa *et al.* (2012)	*TARDBP* (M337V, Q343R, G298S)	TDP-43 protein cytosolic aggregates, co-localized with SNRPB2 (spliceosomal factor); decreased neurite length; increased TDP-43 mRNA levels; cellular vulnerability to arsenite-stressor treatment.	[38]
Sun *et al.* (2018)	*TARDBP* (G298S)	Nuclear and cytoplasmic TDP-43 protein aggregates; NF inclusions; altered mitochondrial axonal transport; increased vulnerability to stress.	[39]
	sALS	Nuclear TDP-43 protein aggregates; NF inclusions; altered mitochondrial axonal transport; increased vulnerability to stress.	
Liu *et al.* (2015)	*FUS* (P525L)	Cytoplasmic mislocalization and formation of FUS protein aggregates.	[40]
Ichiyanagi *et al.* (2016)	*FUS* (H517D)	Cytoplasmic FUS protein mislocalization; presence of stress granules under stress conditions; decreased neurite length; increased cellular vulnerability to oxidative stress and excitotoxicity.	[41]
Naumann *et al.* (2018)	*FUS* (R521C, R521L, R495QfsX527)	Hypoexcitability; impairment of DDR; cytoplasmic FUS protein mislocalization; FUS protein aggregates; increased axonal swelling; loss of mitochondrial membrane potential in distal axons; neurodegeneration.	[42]
Wang *et al.* (2018)	*FUS* (R521H or P525L)	Cytoplasmic FUS accumulation; delayed DNA repair; increased susceptibility to ROS.	[43]

ChAT: Choline-acetyltransferase; DDR: DNA damage response; ER: Endoplasmic reticulum; HSR: Heat-shock response; iPSC: Induced pluripotent stem cell; MN: Motoneuron; NF: Neurofilament; ROS: Reactive oxygen species; UPR: Unfolded protein response.

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Table 1. Major phenotypic alterations detected in motoneurons differentiated from induced pluripotent stem cells of amyotrophic lateral sclerosis patients (cont.).

Study (year)	iPSC line genotypes	Physiological alterations detected in motoneurons *in vitro*	Ref.
Akiyama *et al.* (2019)	*FUS* (p.H517D), generated by knock-in; *FUS* mutation	Aberrant increase of axon branches due to elevated levels of Fos-B mRNA.	[44]
Deshpande *et al.* (2019)	*FUS* (Asp502Thrfs* 27)	Increased postsynaptic accumulation of mutated FUS; presence of protein aggregates.	[45]
Mitne-Neto *et al.* (2011)	*VAPB* (P56S)	Reduction in VAPB protein levels.	[46]
Oliveira *et al.* (2020)	*VAPB* (P56S)	Cell death and mitochondrial oxidative metabolism impairment in MNs derived from severely but not mildly affected patients.	[47]
Burkhardt *et al.* (2013)	sALS	Spontaneous intranuclear and hyperphosphorylated TDP-43 protein aggregates.	[48]
Alves *et al.* (2015)	sALS	Gene ontology indicates mitochondrial dysfunction.	[49]
Fujimori *et al.* (2018)	*FUS* mutation	Lower cell viability; reduced neurite growth and swellings; stress granules; FUS protein cytoplasmic mislocalization.	[50]
	TARDBP mutation	Lower cell viability; reduced neurite growth and swellings; stress granules; TDP-43 protein aggregates.	
	sALS	Lower cell viability; reduced neurite growth; TDP-43 and FUS protein aggregates.	
	C9Orf72 expansion	Increased expression of transcripts related to mitochondrial transport and oxidative stress.	
Osaki *et al.* (2018)	*TARDBP* (G298S)	Increased TDP-43 mRNA expression; decreased NF mRNA; TDP-43 protein aggregates in cytoplasm and nucleus; reduced ChAT expression; cell degradation.	[51]
Hall *et al.* (2017)	*VCP* mutation	TDP-43 protein aggregates; endoplasmic reticulum stress; mitochondrial dysfunction.	[52]

ChAT: Choline-acetyltransferase; DDR: DNA damage response; ER: Endoplasmic reticulum; HSR: Heat-shock response; iPSC: Induced pluripotent stem cell; MN: Motoneuron; NF: Neurofilament; ROS: Reactive oxygen species; UPR: Unfolded protein response.

Table 2. Major phenotypic alterations detected in astrocytes differentiated from induced pluripotent stem cells of amyotrophic lateral sclerosis patients

Study (year)	iPSC line genotypes	Physiological alterations detected in astrocytes *in vitro*	Ref.
Almad *et al.* (2016)	*SOD1* mutation; *C9orf72* expansion; sALS	Cx43 (major astrocyte connexin) upregulation.	[53]
Mohamed *et al.* (2019)	*SOD1* (A4V); *FUS* (H517Q); *C9orf72* expansion; sALS	*SOD1*, *FUS* and sporadic (but not *C9orf72*) derived ACM increases P-gp expression in cultured human endothelial cells due to excess glutamate release.	[54]
Madill *et al.* (2017)	*C9orf72* expansion	ACM reduces viability of cultured MN; ACM impairs autophagy mechanisms and increases SOD1 expression in cultured HEK293T cells.	[55]
Birger *et al.* (2019)	*C9orf72* expansion	Accelerated senescence and production of ROS; ACM reduced ESC-derived cortical-neuron viability; ACM reduced ESC derived-MN viability; altered transcriptome profile, with enrichment for genes involved in cellular senescence pathways; altered secretome, with reduced levels of antioxidants.	[56]
Zhao *et al.* (2020)	*C9orf72* expansion	Intranuclear RNA foci; capable of modifying membrane capacitance of control MNs	[36]
Serio *et al.* (2013)	*TARDBP* (M337V)	Cytoplasmic mislocalization of TDP-43 protein; increased cell death.	[57]

ACM: Astrocyte-conditioned medium; ESC: Embryonic stem cell; iPSC: Induced pluripotent stem cell; MN: Motoneuron; ROS: Reactive oxygen species.

ALS-iPSC-derived MNs

Considering that MNs are the main cell type affected during ALS progression, several groups have differentiated MNs from fALS and sALS iPSCs. Important phenotypic alterations have been detected in MNs obtained from post-mortem tissues of ALS patients, such as protein aggregates, changes in mitochondrial morphology and cell degeneration [63]. MNs derived from iPSCs obtained from ALS patients with different genotypes also feature these alterations, among others, as described below and summarized in Tables 1 & 2.

SOD1–iPSC–MNs

Several studies using *SOD1*-animal models have accumulated evidence on *SOD1*-ALS disease progression, describing numerous dysfunctions and altered pathways. In these models, MNs showed, for example, SOD1 protein aggregates, increased oxidative stress, mitochondrial dysfunction and cytoskeleton-associated protein neurofilament (NF) degeneration, among others [64]. Similar aggregates have been detected in MNs (but rarely in non-MNs)

Table 3. Main cellular alterations found in motoneurons differentiated from induced pluripotent stem cells of amyotrophic lateral sclerosis patients.

Cellular alterations	Mutated gene	Study (year)	Ref.
Reduced cell viability	*SOD1*	Chen *et al.* (2014); Kiskinis *et al.* (2014)	[21,22]
	C9orf72	Dafinca *et al.* (2016); Birger *et al.* (2019)	[31,56]
	TARDBP	Bilican *et al.* (2012); Fujimori *et al.* (2018); Osaki *et al.* (2018); Serio *et al.* (2013)	[37,50,51,57]
	FUS	Fujimori *et al.* (2018); Marrone *et al.* (2019); Japtok *et al.* (2015)	[50,58,59]
	VAPB	Oliveira *et al.* (2020)	[47]
	Sporadic	Fujimori *et al.* (2018)	[51]
Increased susceptibility to different stressors	*C9orf72*	Donnelly *et al.* (2013); Dafinca *et al.* (2016); Selvaraj *et al.* (2018); Shi *et al.* (2018); Lynch *et al.* (2019)	[31,33,34,60,61]
	TARDBP	Egawa *et al.* (2012); Sun *et al.* (2018)	[38,39]
	FUS	Wang *et al.* (2018); Ichiyanagi *et al.* (2016)	[41,43]
	Sporadic	Sun *et al.* (2018)	[39]
Neurite abnormalities	*SOD1*	Chen *et al.* (2014); Kiskinis *et al.* (2014)	[21,22]
	TARDBP	Egawa *et al.* (2012); Fujimori *et al.* (2018)	[38,50]
	FUS	Fujimori *et al.* (2018); Naumann *et al.* (2018); Ichiyanagi *et al.* (2016); Akiyama *et al.* (2019)	[41,42,44,51]
	Sporadic	Fujimori *et al.* (2018)	[51]
Presence of protein aggregates	*SOD1*	Chen *et al.* (2014); Imamura *et al.* (2017); Seminary *et al.* (2018)	[21,25,27]
	C9orf72	Seminary *et al.* (2018); Dafinca *et al.* (2016); Seminary *et al.* (2020); Lynch *et al.* (2019)	[27,31,35,61]
	TARDBP	Seminary *et al.* (2018); Egawa *et al.* (2012); Fujimori *et al.* (2018); Sun *et al.* (2018); Osaki *et al.* (2018)	[27,38,39,50,51]
	FUS	Liu *et al.* (2015); Wang *et al.* (2018); Japtok *et al.* (2015); Naumann *et al.* (2018); Deshpande *et al.* (2019)	[40,42,43,45,59]
	VCP	Hall *et al.* (2017)	[52]
	Sporadic	Fujimori *et al.* (2018); Sun *et al.* (2018); Burkhardt *et al.* (2013)	[39,48,50]
Mitochondrial alterations	*SOD1*	Kiskinis *et al.* (2014)	[22]
	C9orf72	Dafinca *et al.* (2016); Lopez-Gonzalez *et al.* (2016); Lynch *et al.* (2019); Fujimori *et al.* (2018)	[31,32,50,61]
	TARDBP	Sun *et al.* (2018)	[39]
	FUS	Naumann *et al.* (2018)	[42]
	VAPB	Oliveira *et al.* (2020)	[47]
	VCP	Hall *et al.* (2017)	[52]
	Sporadic	Alves *et al.* (2015)	[49]
Electrophysiological changes	*SOD1*	Wainger *et al.* (2014); Naujock *et al.* (2016); Bursch *et al.* (2019)	[23,24,28]
	C9orf72	Wainger *et al.* (2014); Bursch *et al.* (2019); Sareen *et al.* (2013); Selvaraj *et al.* (2018); Zhao *et al.* (2020); Shi *et al.* (2018); Devlin *et al.* (2015)	[23,28–30,33,34,36]
	FUS	Wainger *et al.* (2014); Naujock *et al.* (2016); Bursch *et al.* (2019)	[23,24,28]
	TARDBP	Bursch *et al.* (2019); Devlin *et al.* (2015)	[28,30]
	Sporadic	Naujock *et al.* (2016)	[24]
Number of stress granules or activation of endoplasmic reticulum stress	*SOD1*	Kiskinis *et al.* (2014)	[22]
	C9orf72	Seminary *et al.* (2018); Dafinca *et al.* (2016)	[27,31]
	TARDBP	Fujimori *et al.* (2018)	[50]
	FUS	Fujimori *et al.* (2018); Lenzi *et al.* (2015); Ichiyanagi *et al.* (2016)	[41,50,62]
	VCP	Hall *et al.* (2017)	[52]

iPSC: Induced pluripotent stem cell; MN: Motoneuron.

differentiated from iPSCs obtained from fALS patients harboring two different *SOD1* mutations, D90A and A4V, which also feature phenotypic alterations such as reduced SOD1 protein levels and neurite swellings [21]. In another study, MNs derived from A4V-patients, compared with control MN-derived iPSCs, displayed less viability, reduced soma size and neurite outgrowth, and significant alterations in mitochondrial morphology and motility. Moreover, transcriptome analysis indicated an increased activation of endoplasmic reticulum (ER) stress and unfolded protein response pathways [22]. A more recent study by Wang and colleagues also detected important alterations in the *SOD1*-A272C MN transcriptome, with downregulation of transcripts involved in calcium and ER homeosta-

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sis [26]. Other groups have focused on understanding the relation between protein aggregation and homeostasis pathways. MNs derived from iPSC with *SOD1*- L144FVX accumulate misfolded SOD1 protein and autophagy markers, and are more vulnerable to cell death [25]. Accordingly, Seminary and collaborators demonstrated that, as expected, MNs derived from iPSCs with *SOD1*-N139K also have increased levels of insoluble SOD1 protein, as a result of impaired activation of the heat-shock response to the increased protein burden. However, cell viability was not reduced in relation to control MNs [27]. Regarding plasma-membrane electrical properties, Wainger and collaborators demonstrated in 2014 that MNs from iPSCs with the A4V mutation have intrinsic hyperexcitability, probably due to delayed rectifier potassium-current amplitudes [23]. Conversely, Naujock and colleagues described an opposite phenotype in MNs differentiated from iPSCs harboring D90A and R115G mutations: plasma membrane hypoexcitability, impaired spontaneous activity and reduced sodium-channel levels, leading to imbalanced sodium/potassium-current ratios [24]. These opposing trends may reflect different phenotypes of the donor patients. Recently, increased expression of metabotropic glutamate receptors in the D90A-MNs plasma membrane was also demonstrated, suggesting facilitation of excitatory transmission in these cells [28].

C9orf72–iPSC–MNs

In healthy subjects, hexanucleotide intronic repeats (GGGGCC) in the *C9orf72* gene first intron usually range between 3 and 30. ALS and FTD patients with alterations in this gene, on the other hand, have hundreds to thousands of repetitions [4]. Three mechanisms have been proposed as responsible for the pathological effects induced by this expansion: haploinsufficiency of C9 protein, encoded by this gene and the physiological function of which is not yet fully understood; formation of nuclear expanded RNA foci, which sequester nuclear RNA-binding proteins; and increased RAN-translation of dipeptide repeat proteins (DPRs), which are prone to form cytosolic aggregates [65]. A reduction in C9orf72 RNA levels has been detected in mixed neuron cultures derived from ALS-iPSCs with expansions in this gene, along with altered gene expression and increased susceptibility to excitotoxicity [60]. C9 protein levels were also decreased in MNs derived from ALS/FTD-iPSCs [29]. However, C9 haploinsufficiency was not detected in MNs differentiated from *C9orf72*-ALS-iPSC lines in other studies discussed in this section. This difference may reflect different numbers of expansions in the donors, or this reduction may be more significant in non-MN neuron cells, as post-mortem analyses of ALS/FTD patients' brains also indicated a reduction in C9 mRNA levels [4]. RNA foci were repeatedly detected in MNs [29,31–33,36] and in mixed neurons [60] derived from *C9orf72*-ALS-iPSC lines, as well as DPRs [29,31,65]. Moreover, some groups have described a reduction in cell viability or increased susceptibility to excitotoxicity in relation to MNs derived from control iPSC lines, possibly related to elevated expression of Ca^{2+}-permeable glutamatergic ionotropic receptors [31,33,34]. As in S0D1–iPSCs–MNs, controversial changes in cellular excitability have also been described: Sareen and colleagues detected hypoexcitability of the MN plasma membrane [29], while Wainger and collaborators reported signs of hyperexcitability [23]. Interestingly, in a 2015 contribution, Devlin helped to clarify these inconsistencies: MNs initially show signs of hyperexcitability but in long-term cultures there is a progressive loss of action-potential output and perturbations in Na+/K+ currents, resulting in reduced synaptic activity [30]. Also, it was recently described that MNs feature detectable spontaneous transient electrical signals concomitantly with elevated expression of glutamate-kainate receptors, voltage-gated Ca^{2+} channels, and choline-acetyltransferase enzymes [28]. *C9orf72*-MNs also showed alteration in mitochondrial morphology and membrane potential, increased ER stress, and dysfunction in Ca^{2+} homeostasis [31], along with elevated reactive oxygen species production [32]. Regarding protein aggregates, levels of insoluble SOD1 and TDP-43 tend to be increased in *C9orf72*-MNs [27,35]. Finally, *C9orf72*-skeletal myocytes also feature several phenotypic alterations, such as uncoordinated contractions, RNA foci, DPRs, modifications in mitochondrial-gene expression, increased susceptibility to oxidative stress and aggregation of phosphorylated TDP-43 protein [61].

TARDBP–iPSC–MNs

TDP-43, the protein encoded by the *TARDBP* gene, is the main constituent of protein aggregates found in neural cells from the motor cortex and spinal cord of sALS and some fALS patients [66]. TDP-43 is related to RNA metabolism, participating in RNA binding, transport and suppression of aberrant splicing. It is not yet understood whether the pathological effects of mutated TDP-43 are due to a toxic gain of function or to the reduction of soluble nuclear TDP-43 levels [67]. The presence of TDP-43 nuclear or cytoplasmic aggregates is a constant feature of MNs differentiated from ALS-iPSCs harboring different *TARDBP* mutations [27,37–39,50]. Several groups have also described reduced MN viability [37,50] or increased vulnerability to different stressors, such as arsenite or

proteasome inhibitors [38,39]. Interestingly, Fujimori and colleagues reported the presence of stress granules and neurite swellings as well as a reduction in neurite growth [50]. In agreement, Egawa and co-workers also detected a significant reduction in neurite length [38]. *TARDBP*-MNs harboring the M337V mutation showed a significant alteration in membrane excitability and a progressive reduction in synaptic activity [30], as well as increased Ca^{2+} levels and AMPA receptor-induced signal amplitudes. Altered mitochondrial-axon transport and the presence of cytoplasmic NF inclusions were other important phenotypic alterations described in MNs with the *TARDBP*-G298S mutation [39]. Using an interesting *in vitro* 3D model of the motor unit, Osaki and colleagues showed that MNs harboring the *TARDBP-G298S* mutation have increased TDP-43 mRNA expression and protein aggregation in the cytoplasm and nucleus, as well as decreased NF mRNA and choline-acetyltransferase expression. Moreover, muscle fibers featured fewer contractions and increased apoptosis rates [51].

FUS–iPSC–MNs

The FUS protein, similarly to TDP-43, is also involved in nuclear RNA metabolism. In ALS patients with a mutation in this gene, the presence of cytoplasmic FUS aggregates is an important histopathological hallmark. A rare *FUS*-P525L missense mutation is responsible for an aggressive early-onset ALS form [68]. In ventral spinal-cord neural cells differentiated from iPSCs harboring this mutation, generated by TALEN-directed mutagenesis, cytoplasmic FUS protein is detectable in stress granules upon oxidative stress, a phenotypic alteration also detected in cells differentiated from iPSCs obtained from fALS *FUS* patients [62]. Recently, similar alterations were detected in spinal neurons derived from iPSCs, which also displayed increased apoptosis and deficits in protein degradation in a stress-dependent manner [58]. FUS cytoplasmic aggregates were found in MNs derived from iPSCs of a 22-year-old P525L patient [40]. Wang and colleagues also described the presence of cytoplasmic FUS accumulation in MNs derived from two different *FUS* patients, one of whom harbored the P525L mutation, along with delayed ability for DNA repair and increased susceptibility to oxidative stress [43]. The presence of mislocalized cytoplasmic FUS aggregates is a common feature of non-MN neuronal cells differentiated from iPSC lines harboring other *FUS* mutations. In cortical neurons, besides cytoplasmic protein aggregates, another important alteration was a reduction in viable cells in aged cultures [59]. In MNs, increased neuronal death *in vitro* was also reported by Naumann and collaborators, along with impaired DNA-damage response, axonal swelling and an alteration in mitochondrial potential [42]. Structural alterations in neurites were also described by Ichiyanagi and co-workers, who found decreased neurite length as well as increased neuronal vulnerability and formation of granules after stress induction [41]. Interestingly, although most groups showed reduced neurite length, an increase in axonal branching was also seen in MNs [44]. Finally, significant alterations in MNs membrane excitability were described by several groups. Initial reports indicated a tendency to hyperexcitability [23], but another study suggested membrane hypoexcitability, along with alteration in Na+ and K+ voltage-gated channels [24]. Recently, an increase in ionotropic glutamate receptors was also detected [28], as well as augmented postsynaptic accumulation of mutated FUS protein [45].

Other genes–iPSC-MNs

IPSCs have also been derived from ALS patients harboring less-frequent genetic mutations, in genes such as *VAPB* and *VCP*. The *VAPB* gene encodes an integral membrane protein involved in several physiological functions, such as formation of presynaptic terminals and unfolded protein responses, but very little information has been reported regarding *VAPB*-ALS-derived iPSCs. Mitne-Neto and colleagues generated iPSC lines from four fALS patients harboring the P56S mutation. P56S-MNs did not feature cytoplasmic protein aggregates or accumulation of ubiquitinated proteins, typical hallmarks found in ALS patient MNs [69]. However, a downregulation in VAPB protein levels was detected [46]. A recent report using iPSCs derived from five P56S-*VAPB* patients from the same family but with different clinical courses demonstrated that *in vitro* cell death and impairment of the mitochondrial oxidative metabolism were significantly elevated in MNs derived from severely, but not from mildly affected patients [47]. Recently, our group also described derived iPSC lines from two Brazilian *VAPB* patients, one from a P56S-fALS and the other from an sALS, harboring the single nucleotide polymorphism rs2234487 [70]. Further experiments are now being performed to differentiate and characterize MNs and astrocytes derived from these iPSCs. *VCP*, responsible for 2% of fALS cases [71], encodes a valosin-containing protein, which is related to cellular functions such as protein homeostasis and mitochondrial quality control [72]. VCP–iPSCs–MNs feature cytoplasmic TDP-43 protein aggregates and increased ER stress, as well as mitochondrial dysfunction [52]. IPSCs from patients with mutations in other genes such as *CHMP2B* [73] and *ANG* [74] have also been derived. Phenotypic alterations in MNs differentiated from these cell lines have not yet been described.

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sALS–iPSC–MNs

Due to the absence of animal models, modeling the sporadic form of ALS has been a challenge. However, post-mortem analysis of patient tissues provided useful insights about MN pathology in sALS, for instance, the presence of TDP-43 cytoplasmic aggregates [75]. Similar nuclear and cytosolic protein aggregates were detected in MNs and forebrain cortical neurons differentiated from sALS-iPSC lines [39,48,50]. FUS-immunoreactive inclusions were also detected in spinal anterior-horn neurons from sALS patients [70], and similar inclusions were detected in a large screening by Fujimori and colleagues, who used iPSC-MNs derived from 32 sALS patients. These MNs also featured reduced viability and neurite growth. Interestingly, the MN alterations were proportional to the disease progression rate in the donor patient [50]. Other important phenotypic alterations in MNs were described by Sun and collaborators, such as NF inclusions, increased neuronal vulnerability to proteasome inhibitor-induced stress, and altered mitochondrial transport in neurites [39]. Regarding mitochondrial metabolism, microarray analyses have indicated that mitochondrial dysfunction might be associated with MNs neurodegeneration [49]. Finally, impaired spontaneous activity was also described in sALS-MNs, as well as alterations in electrical properties of the plasma membrane [27].

ALS iPSC-derived astrocytes

Accumulating evidence from ALS animal models indicates that glial cells, particularly astrocytes, play an important non-cell deleterious role in MN degeneration [76]. Important phenotypic alterations described in astrocytes derived from ALS-iPSCs are summarized in Table 2. *TARDBP*-astrocytes showed cytoplasmic mislocalization of the TDP-43 protein and reduced cell viability, but no pathogenic effect was exerted in co-cultured MNs [57]. However, recent studies have found significant toxic effects of astrocytes on MNs *in vitro*. *SOD1* and *C9orf72*-astrocytes have increased levels of Cx43, the major astrocyte gap-junction connexin, important for astrocyte interconnection and homeostatic functions. Cx43 upregulation seems to contribute to elevated intracellular Ca^{2+} levels in astrocytes, which could be related to MN degeneration in co-culture [53]. *C9orf72*-astrocyte-conditioned medium (ACM) reduces MN viability, impairs autophagy and increases SOD1 expression in cultured HEK293T cells [55]. Recently, *C9orf72*-astrocytes were found to show accelerated senescence and production of reactive oxygen species. In addition, their ACM reduced embryonic stem cell-derived-cortical neuron viability due to a decrease in trophic support; while reduced embryonic stem cell-derived MN viability was due to a nonidentified secreted toxic factor [56]. Using an *in vitro* model of the blood–brain barrier, Mohamed and colleagues also demonstrated that ACM from iPSCs harboring different mutations increases the expression of the multispecific drug-efflux transporter P-glycoprotein in cultured human endothelial cells. These data suggest a reduction in blood–brain barrier permeability to drugs, a condition also detected in ALS patients in late stages of the disease [54]. Finally, in a recent report, Zhao and colleagues detected the presence of intranuclear RNA foci in *C9orf72* astrocytes, which also feature the ability to modify the capacitance of the co-cultured MN membrane [36].

iPSC-ALS modeling limitations & challenges

The substantial evidence discussed in previous sections demonstrates that neural cells, such as MNs and astrocytes, differentiated from iPSC lines derived from familial or sporadic ALS patients do recapitulate several phenotypic alterations initially described in post-mortem tissues of patients, which will be discussed in more detail in the next section. Although the genes classically associated with ALS pathology encode proteins related to different cell functions and signaling pathways, which may be related to the different clinical forms and progression of ALS, the final result is invariably the same: MN degeneration. However, the results of different research groups regarding the viability of MNs differentiated from ALS-iPSCs are still quite contradictory: some lines of ALS-MNs showed a significant reduction in viability in relation to MNs differentiated from control iPSCs, while others did not display this important difference (Tables 1 & 3). These variations can result from different factors, such as donor genotypes, MN maturation time and the use of different protocols for iPSC generation and MN differentiation.

Regarding the genetic background, even among cases of fALS, some mutations are more aggressive than others, being related to early onset ALS and accelerated disease progression, for example, as reviewed by Gros-Louis and colleagues [77]. Therefore, it is possible that MNs harboring certain mutations require longer culture times for significant changes in viability to be detected. Interestingly, the large-scale study recently conducted by Fujimori and colleagues using 32 sALS-iPSCs demonstrated a significant correlation between the disease progression rate, but not age of onset, and the appearance of phenotypic changes in MNs *in vitro* [50]. These findings indicate that

the clinical variability found in patients with ALS indeed appear to also result in important variations in cellular behavior *in vitro*, which may be quite interesting considering the current need for a more personalized medicine.

Other factors that may account for the variability in results are the different culture conditions and differentiation protocols used. IPSCs can be obtained through different reprogramming methods, and from different adult tissues, such as blood, skin or even urine [78]. This wide methodological variability may possibly contribute to the relatively low reproducibility of studies using neural cells differentiated from these iPSCs. Thus, the use of standardized protocols in large-scale studies is necessary to validate these findings. Moreover, the reprogramming process may also result in partial or total loss of epigenetic changes [79], and genetic mutations can accumulate in iPSCs during their generation or after successive passages [80]. Therefore, comparison of phenotypic characteristics between different cell lines is not a simple matter. An alternative to minimize this problem is the use of isogenic cell lines subjected to mutation correction by genome-editing techniques such as CRISPR/Cas after reprogramming, a strategy adopted in several reports summarized in this review.

Another technical aspect that varies substantially among studies using iPSCs is the protocol used to differentiate the neural cells. Since the work of Dimos and collaborators in 2008, which demonstrated for the first time that it was indeed possible to differentiate MNs from iPSCs derived from an ALS patient [17], these protocols have been continually improved in order to generate the cell type of interest more efficiently. The currently described protocols differ mainly in the stages of neural induction and maturation, resulting in MN cultures with different degrees of purity [81]. ALS has an important nonautonomous cellular component, and astrocytes appear to play an important role in the progression of the disease. Thus, contaminating neural cells could have important effects, both in MN generation and in the purity of their phenotype. In addition, different clones derived from the same individual may show phenotypic variability that could be a result of desynchronized differentiation *in vitro*, forming a heterogeneous MN population. An example of this issue was described by Egawa and collaborators, in which both aggregation of TDP-43 and oxidative stress induced by cell death varied substantially among MNs derived from different iPSC clones from the same patient [38].

Another important issue is that iPSCs have characteristics of embryonic cells, and the reprogramming process has been associated with increased telomere length [82], mitochondrial rejuvenation [83] and loss of senescence markers [84], hampering the evaluation of late-onset cellular alterations such as those typically seen in ALS patients. An interesting strategy that has been used to overcome this limitation is the use of pharmacological agents to accelerate the maturation of differentiated neural cells. One of these agents is progerin, a truncated form of laminin produced in the Hutchinson-Gilford progeria syndrome, characterized by premature aging of several body tissues. Short-term progerin exposure induces aging-related markers in Parkinson's disease iPSC-derived neurons, including dendrite degeneration and mitochondrial swelling [85]. This approach, however, has not yet been tested in ALS-iPSCs. Finally, several studies have demonstrated that when MNs derived from iPSCs that initially did not show significant differences in viability from their respective isogenic controls were exposed to different types of stressors, they were more sensitive, as listed in Table 3. Therefore, stress induction may be a useful approach to accelerate the onset of important phenotypic alterations *in vitro* in nonaged MNs.

Translational perspective

Theoretically, MNs differentiated from autologous or allogeneic iPSCs could be used therapeutically to replaced damaged neurons in neurodegenerative diseases. Recently, the first report of dopaminergic progenitor cells differentiated from iPSC implantation in a Parkinson's disease patient was published [86]. In ALS, however, the long distance that the axon must travel to reach the target and properly form a synapse remains an important obstacle, and so replacement cell therapy is still a distant hope. Recently, the 3D-model of the motor unit derived from iPSCs, developed by Osaki, Uzel and Kamm, appeared to be a promising platform to evaluate axonal regeneration in the context of ALS [51]. Nevertheless, despite the current limitations and technical challenges discussed in the previous section, iPSC lines derived from ALS patients, and especially the cells differentiated from these lines, are a useful and promising method of disease modeling, especially for the ALS sporadic form, for which no other preclinical model is available to date.

In order to validate this approach, it is important to ensure that the main ALS histopathological markers are in fact reproduced in these cell lines. ALS diagnosis is extremely difficult, relying only upon clinical signs. However, post-mortem analyses of tissues from ALS patients, especially the spinal cord, motor cortex and skeletal-muscle cells, indicate that the presence of intracellular protein aggregates is an important hallmark of this disease [9]. Cytoplasmic TDP-43 inclusions are present in fALS patients who harbor mutations in *TARDBP* and in other

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genes, with the exception of *SOD1*, and also in sALS [75]. Using MN-derived iPSCs, the presence of these aggregates is constantly detected in *TARDBP*-MNs [27,37–39,50,51] and in sALS-MNs [39,48,50]. Cytoplasmic FUS protein mislocalization has also been detected in autopsies of ALS patients with mutations in the encoding gene of this protein, and are also consistently present in MNs differentiated from iPSCs of FUS patients [40–43,45,50,59]. In tissues of SOD1-fALS patients, SOD1 intracellular aggregates are a recurrent feature [87]. However, in *in vitro* studies using MNs differentiated from iPSCs, the presence of SOD1 cytoplasmic inclusions is still controversial, and has been described by some groups [21,25,27] but not by others [22]. In *C9orf72*-fALS patients, post-mortem analysis demonstrated the presence of three important and exclusive pathological markers: intranuclear RNA foci, DPRs and TDP-43 cytoplasmic inclusions [88]. Intranuclear RNA foci and DPRs were detected constantly in *C9orf72*-MNs [29,31–33,60]. As for the TDP-43 cytoplasmic inclusions, some groups have reported the presence of cytoplasmic protein aggregates in *C9orf72*-MNs [31,35,89]. Therefore, the presence of different types of intracellular protein inclusions in MNs differentiated from ALS iPSCs appears to be a potential marker for validating these cell lines for modeling ALS in basic and preclinical studies and for large-scale drug screenings.

Recently, more than ten different studies using iPSC models identified at least 25 drugs as potential candidates for ALS therapy [90], and some are now being tested in clinical trials. For instance, Bosutinib, an Src/c-Abl inhibitor initially approved for treating chronic myelogenous leukemia, is currently being repurposed for ALS in a Phase I clinical trial named iDReAM (iPSC-based Drug Repurposing for Amyotrophic lateral sclerosis Medicine) [91]. Bosutinib treatment increased the survival of MNs differentiated from a fALS-*SOD1* iPSC line, ameliorated impaired autophagy and reduced the levels of misfolded SOD1 and TDP-43. Remarkably, these findings were also reproduced in *TARDBP*, *C9orf72* and sALS-MNs [25]. Ropinirole, a D2 and D3 dopamine receptor agonist, which is usually used as an antiparkisonian drug, showed several promising results *in vitro*, such as suppression of oxidative stress, improvement of mitochondrial function, and inhibition of protein aggregation and cell death. Interestingly, these effects were observed only in non-SOD1 cell lines, reinforcing the importance of patient stratification in clinical studies [50]. The anti-epileptic drug retigabine, a voltage-gated potassium-channel activator, on the other hand, was able to reduce MN excitability and death in cells harboring mutations in the *SOD1*, *FUS* and *C9orf72* genes [23]. Therefore, the ability to model ALS pathophysiology *in vitro* can also be useful for drug screening, contributing to the search for therapies in a patient-specific manner. This requires a large number of neural cell lines differentiated from ALS iPSCs with varied genetic backgrounds. Answer ALS (https://www.answerals.org/) is a global project aiming to generate unique iPSCs from over 1000 ALS patients and healthy controls. It is expected that in the near future, representative cell lines from most of the different ALS genotypes and phenotypes will be available, which will be extremely useful in large-scale drug screenings and to increase our understanding of the pathophysiological mechanisms of this disease, which still remain elusive.

Author contributions

JF Vasques and F Gubert performed the literature search and data assembling; JF Vasques drafted the manuscript; R Mendez-Otero and F Gubert critically revised the work.

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Executive summary

Background

- Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease that mainly affects motoneurons (MNs), leading to muscle atrophy and death from paralysis of the respiratory muscles.
- The two drugs available for ALS patients extend life expectancy by only a few months.
- ALS is classified as familial (fALS) or sporadic (sALS), and mutations in several genes, such as *SOD1*, *C9orf72*, *TARDBP* and *FUS*, have been related to fALS.
- ALS pathology varies among patients with different genetic backgrounds. Animal models harboring specific mutations do not represent the pathophysiology of all patients, which is related to the failure of clinical trials of drugs tested in preclinical studies.
- Neural cells differentiated from induced pluripotent stem cells (iPSCs) of ALS patients are promising tools for disease modeling and drug screening in a more-specific manner.

ALS-iPSC-derived MNs

- Several phenotypic alterations detected in post-mortem tissues of ALS patients were reproduced in ALS-iPSC-derived MNs, including protein aggregates, changes in mitochondrial morphology and cell degeneration.
- *S0D1*–iPSC–MNs feature abnormalities such as SOD1 protein aggregates, neurite swelling, reduced viability and altered electrical properties, although some of these findings were not reproduced in all studies.
- *C9orf72*–iPSC–MNs often showed alterations in C9 protein levels, RNA foci and dipeptide repeat proteins, which are prone to aggregate. Some research groups have also detected alterations in excitability, susceptibility to excitoxicity and cell viability.
- *TARDBP*–iPSC–MNs consistently feature TDP-43 aggregates as well as reduced viability and increased vulnerability to different stressors. Alterations in neurite length and synaptic transmission have also been described in some lines.
- *FUS*–iPSC–MNs commonly show cytoplasmic FUS protein aggregates. Alterations in cell viability, susceptibly to stress and membrane electrical properties have also been reported.
- *VAPB*–iPSC–MNs showed reduced levels of VAPB protein, and mitochondrial oxidative metabolism was impaired, according to disease severity.
- sALS–iPSC–MNs feature important histological markers of sALS patients, such as TDP-43 or FUS protein aggregates, and cell viability seems to be proportional to a patient's rate of disease progression.

ALS iPSC-derived astrocytes

- Important phenotypic alterations have been described in astrocytes derived from ALS-iPSCs, such as cytoplasmic protein aggregates and reduced cell viability. Toxic effects of ALS iPSC-derived astrocytes on MNs *in vitro* vary among the reports analyzed.

iPSC-ALS modeling limitations & challenges

- Phenotypic alterations vary widely among iPSC-derived neural cells, probably due to the donor's genetic background or variations in the differentiation protocols. Better-standardized protocols are necessary to increase reproducibility.

Translational perspective

- Neural cells differentiated from ALS patients' iPSCs are a promising method of disease modeling, especially for the sporadic form of ALS. They are also a potential tool for *in vitro* drug screening, stratifying responder and nonresponder patients.

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