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Review

The Role of Human Pluripotent Stem Cells in Amyotrophic Lateral Sclerosis: From Biological Mechanism to Practical Implications

Laura Ceccarelli[1,2,†], Lorenzo Verriello[3,†], Giada Pauletto[3], Mariarosaria Valente[1,2], Leopoldo Spadea[4], Carlo Salati[5], Marco Zeppieri[5,*], Tamara Ius[6]

[1]Clinical Neurology Unit, Head-Neck and Neurosciences Department, Santa Maria della Misericordia University Hospital, ASUFC, 33100 Udine, Italy
[2]Department of Medicine (DMED), Santa Maria della Misericordia University Hospital, ASUFC, 33100 Udine, Italy
[3]Neurology Unit, Head-Neck and Neurosciences Department, Santa Maria della Misericordia University Hospital, ASUFC, 33100 Udine, Italy
[4]Eye Clinic, Policlinico Umberto I, "Sapienza" University of Rome, 00185 Rome, Italy
[5]Department of Ophthalmology, University Hospital of Udine, 33100 Udine, Italy
[6]Neurosurgery Unit, Head-Neck and Neurosciences Department, Santa Maria della Misericordia University Hospital, ASUFC, 33100 Udine, Italy
*Correspondence: markzeppieri@hotmail.com (Marco Zeppieri)
[†]These authors contributed equally.

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder, characterized by progressive loss of both upper and lower motor neurons, resulting in clinical features such as muscle weakness, paralysis, and ultimately, respiratory failure. Nowadays, there is not effective treatment to reverse the progression of the disease, that leads to death within 3–5 years after the onset. Nevertheless, the induced pluripotent stem cells (iPS) technology could be the answer, providing disease modelling, drug testing, and cell-based therapies for this pathology. The aim of this work was to conduct a literature review of the past 5 years about the role of iPS in ALS, to better define the neurobiological mechanisms involved in the pathogenesis and the potential future therapies. The review also deals with advanced and currently available technologies used to reprogram cell lines and generate human motor neurons *in vitro*, which represent the source to study the pathological processes, the relationship between phenotype and genotype, the disease progression and the potential therapeutic targets of these group of disorders. Specific treatment options with stem cells involve Advance Gene Editing Technology, neuroprotective agents, and cells or exosomes transplantation, aimed to replace dead or damaged nerve cells. In summary, this review comprehensively addresses the role of human pluripotent stem cells (hPSCs) in motor neuron diseases (MND), with a focus on physiopathology, diagnostic and prognostic implications, specific and potential future treatment options. Understanding the biological mechanisms and practical implications of hPSCs in MND is crucial for advancing therapeutic strategies and improving outcomes for patients affected by these devastating diseases.

Keywords: stem cells; ALS; motor neuron disease; neurodegenerative disease; human induced pluripotent stem cell (hiPSC); cell therapy; amyotrophic lateral sclerosis; drug screening; disease models

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a lethal neurodegenerative disease of the central nervous system characterized by the progressive and relentless loss of both upper and lower (cortical and spinal) motor neurons. First described in 1860 by Jean-Martin Charcot, its currently clinical presentation is well defined by a plethora of signs and symptoms. Typically, it starts with focal muscle weakness and atrophy in the distal limbs, which later progresses until it involves bulbar muscles, ultimately leading to paralysis of respiratory muscles and resulting in patient's death within approximately 3–5 years from the onset of the disease.

Standardized global ALS incidence by meta-analysis is 1.68 per 100,000 person-years, but varies by region [1] and the average age of onset is 64 years. The most common cases (about 90%) are sporadic forms sporadic amyotrophic lateral sclerosis (sALS), which occur spontaneously without a well-identified cause, while a small portion (approximately 10%) are familial forms (fALS) [2], defined for patients with a positive family history, for which currently over 40 genes have been identified in the pathogenesis of the disease. The most studied and well-defined disease-causing genetic variant in experimental models is the point mutation of the *SOD1* gene that encodes for the enzyme superoxide dismutase. Discovered in 1993 [3], it causes the accumulation of misfolded protein and increased oxidative stress in neurons, hyperexcitability and mitochondrial dysfunction leading to cell death [4]. Another widely described genetic variant, in cases of fALS, is the hexanucleotide GGGGCC expansion in the intron of chromosome nine open reading frame 72 (C9orf72) gene. Transcription of the aberrant gene leads to the formation of pathological RNA, which accumulates in the nucleus and hinders gene transcription [5].

This gene has also been found in cases of ALS associated with Frontotemporal Dementia (FTD): these two pathologies indeed share neuropathological and clinical features. In fact, approximately 40% of FTD patients and 97% of ALS patients exhibit an accumulation of the TDP43 protein. Furthermore, a clinical overlap has been highlighted, with about 5% of diagnosed ALS patients showing behavioral alterations and executive dysfunctions similar to the behavioral variant of FTD. These findings suggest possible common therapeutic targets between the two neurodegenerative conditions [6,7].

Other mutations associated with ALS include TARDP and FUS. However, the etiology of ALS remains unknown in the majority of cases, and its pathogenesis is multifactorial [7–9].

Currently, ALS remains incurable. Treatment is focused on using therapies that might slow disease progression. Moreover, coordinated multidisciplinary treatment represents the standard of care, as it guarantees an increase in survival and improvement in quality of life [9–11].

Some countries have approved a few medications to help the slowing down of disease progression, which include Riluzole and Edaravone [9].

Riluzole is an anti-glutamatergic agent and improves survival by few months [12].

Edaravone, a free radical scavenger and antioxidant, decreases the effects of oxidative stress in ALS and shows some efficacy in *post hoc* analysis of the first phase 3 trial [13]. The trial design, however, does not permit the result of the study to be generalized to all ALS patient population. Questions remain about the drug's effectiveness, which have been raised after post post-marketing observations [14,15].

Tofersen, a novel antisense oligonucleotide (ASO) drug, has recently been approved by the FDA, in April 2023. This drug was approved for the treatment of ALS patients with *SOD1* mutation. In a double-blind, placebo-controlled clinical trial, treatment with Tofersen has shown reduced levels of SOD1 proteins in the cerebrospinal fluid by 36%, in patients with this type of ALS. Earlier treatment with Tofersen appeared to provide better measurement outcomes [16].

The combination of sodium phenylbutyrate and taurursodiol (Relyvrio) has been shown to be effective in reducing neuronal cell death and oxidative injury by reducing mitochondrial dysfunction and oxidative stress in the endoplasmic reticulum. The efficacy of Relyvrio was demonstrated in a randomized, placebo-controlled, multicenter, double-blind, parallel-group study [17]. The study reported that the patients treated with Relyvrio experienced a slower rate of decline in the assessment of daily activities when compared with those treated with placebo.

However, Relyvrio and Tofersen must be further studied, because data are lacking to validate their effectiveness [18].

The past failures of various therapeutic strategies to modify the pathology natural history and the ongoing strong urgency to find new ones, are due to the lacking of precise disease models to conduct research on.

Over the years, numerous *in vitro* and *in vivo* models have been proposed, with the aim of understanding the mechanism of motor neuron degeneration in patients with ALS. Despite significant progress, especially in demonstrating the key role of non-neuronal cells, such as astrocytes and glial cells, in inducing a proinflammatory environment that triggers and accelerates motor neuron death, the pathophysiological process of the disease has not been fully elucidated yet [19].

The difficulty in well understanding the disease arises from its etiological heterogeneity, which is also shared with other neurodegenerative diseases. Among the causes, both genetic and environmental factors are recognized, demonstrating a multifactorial process. Additionally, the complex interactions between neurons and other cells types within the microenvironment, makes it challenging to establish reliable *in vitro* models.

Traditionally, researches, in the context of neurological diseases, have centered around the utilization of animal models, predominantly rodents. Animal models have been a crucial tool in understanding the pathogenesis of ALS too, discovering the cellular and molecular interactions present within the neuromuscular tissue. However, there are lots of constraints associated with employing animal models for studying the disease, which are, for instance, different epigenomic background between humans and other species, different cellular subtypes comprising the brain, and different neuronal networks within the brain. The considerable distinction in brain size is noteworthy, given that human neurons must extend longer axons, transport vesicles and protein machinery across greater distances, and, on average, establish a significantly higher number of synapses per neuron compared to mouse neurons. Moreover, these animal models do not completely replicate the pathology observed in human diseases and, consequently, frequently exhibit limited predictability of drug efficacy in clinical trials [20–22]. In ALS studies, the majority of murine models have relied on the induced overexpression of SOD1 mutated gene, focusing preclinical studies on a model that represents only a small slice of ALS cases and does not molecularly replicate TDP-43 aggregates, defined as a recurrent pathological marker in ALS. Furthermore, there has been observed a poor correspondence between therapeutic results obtained in the preclinical trials on animals and those achieved in humans, suggesting interspecies variability and different patterns of connections in the motor system that make these models less reliable and poorly translating from animals to humans [23].

In fact, despite hundreds of clinical trials based on the discoveries made on animal models, none have allowed for the detection of a significant therapy able to alter the course of ALS.

Another aspect to consider, which may explain why past efforts have been futile, is the challenge of obtaining human nerve cells. For centuries, the study of the cerebral nervous system has indeed been limited by the inability, except through highly invasive and risky procedures, to collect material for analysis.

Examining *post-mortem* tissue from ALS patients offers insight into the full range of pathological phenotypes, even if it only shows a static moment at the disease terminal stage. Not all motor neurons (MNs) respond equally to degeneration in ALS, and the surviving cells observed during autopsy may represent groups relatively resilient to the disease progression. Examination of *post-mortem* tissue samples from ALS patients and murine ALS models has identified the activation of astrocytes and microglia, that confirms that neuroinflammation plays a pivotal role in ALS [24].

More than ten years ago, with the advent of induced pluripotent stem cell technology from adult somatic cells, there has been a revolution in the field of Biology and Medicine. They could be an opportunity to circumvent and overcome these issues and provide the crucial answer to the need for more robust preclinical models in neurodegenerative diseases, including ALS. Thanks to their ability of multipotency, self-renewal and genetic memory, they seem to be the ideal candidate to address the lack of representative ALS disease models [25].

This review aims to providing a comprehensive overview of induced pluripotent stem cells use in ALS, starting from the development of differentiation protocols into various cellular subtypes, and then showing the potential clinical-therapeutic applications discussed and experimented in the literature.

2. Materials and Methods

This review focused on the molecular aspects and clinical implications of iPS in motor neuron diseases. An extensive literature search was conducted through a comprehensive query to identify relevant and original reports describing the clinical application of induced pluripotent stem cells in ALS. The major databases for medical literature were searched (MEDLINE, Web of Science, Cochrane Library, Scopus, EMBASE).

According with the research enquiry, the inclusion criteria were defined as follows: 5-year time range (2018 to 2023), only studies presenting clinically relevant applications of induced pluripotent stem cells were selected, including original research consisting of clinical trials, prospective cohort studies, case series, articles and reviews. Only full-length publications were considered for this research using the search string “induced pluripotent stem cells” AND “motor neuron disease” (64 results) or “stem cells” AND “ALS” (124 results) or “iPSC” AND “Amyotrophic Lateral Sclerosis” (83 results). Some articles were also derived from bibliography of the chosen articles. Papers that were not in English, Italian or French language, outside the scope of this review and duplicates, were excluded. A minimum of two of the authors reviewed each study for relevance. Data extraction and synthesis will be performed to provide a cohesive overview of the current state of knowledge in this field.

3. iPSCs as an Advance on Human Disease Models

Over the years, induced pluripotent stem cells (iPSCs) have been recognized as a valuable resource for analyzing the pathogenetic mechanisms underlying neurodegenerative diseases. They have provided a deeper understanding of the molecular and cellular dysfunctions that manifest in diseased tissues. Moreover, iPSCs have become an invaluable tool in drug experimentation, not only for assessing efficacy and side effects, but also for discovering potential new therapeutic targets.

The applications of stem cells in the field of neurodegenerative diseases, specifically in motor neuron diseases, are manifold [26–29]. They include delving into the pathogenesis by studying different disease phenotypes and genotypes at various stages of progression. Stem cells are crucial tools in identifying new therapeutic targets, testing novel treatments [30], stratifying disease forms based on therapeutic response rather than clinical parameters, and ultimately creating patient-tailored therapies in the realm of precision medicine, often referred to as “Disease in a Dish”.

iPSCs possess the unique ability to self-renew, making them an endless source of cellular material. Additionally, they can differentiate into various cell subtypes, depending on the chosen culture medium and growth conditions. In contrast to embryonic stem cells (ESCs) derived from 4–5-day blastocysts, iPSCs are obtained from adult somatic cells of the patient, which are reprogrammed and returned to a pluripotent status through the ectopic expression of embryonic transcription factors. This approach reduces ethical issues and significantly lowers the risk of immunological rejection when transplanted *in vivo*. Furthermore, these cells retain the genetic background of the donor, revolutionizing the study of neurodegenerative diseases, particularly neuromuscular disorders, by allowing the *in vitro* reconstruction of the genotypic and phenotypic variability of affected patients [31,32].

However, challenges still persist in this technology. In comparison to ESCs, iPSCs may exhibit more frequent epigenetic and genetic aberrations due to the reprogramming process. The considerable variability among cell lines, maintaining the individual genetic heritage of a specific patient, makes it challenging to standardize culture techniques and makes various study models less comparable [33,34].

To obtain pure cell lines independent of genetic variability, one possibility is to use CRISPR/Cas9 technology. It allows for editing the genome of stem cells and precisely modifying specific DNA sequences. The system uses guide RNA to direct the Cas9 enzyme to the target sequence in the DNA, where it induces cuts or repairs mutations. This enables the generation of isogenic cell lines, meaning they share the same modified gene sequences. This genetic manipulation allows for obtaining cell lines with similar and easily identifiable phenotypes, making studies more comparable within the same cell culture or on cell lines obtained from parallel differentiations [35].

Over a decade ago, Takahashi and his team achieved a revolutionary milestone as they successfully, using four embryonic transcription factors (OCT4, SOX2, KLF4, and c-MYC), converted mouse fibroblasts into induced pluripotent stem cells (iPSCs). Since then, reprogramming techniques and cellular differentiation models have evolved, and today it is possible to obtain pluripotent stem cells (PSCs) from three germ cell types, originating from each embryonic germ layer: fibroblasts (mesoderm-derived), hepatocytes (endoderm-derived), and keratinocytes (ectoderm-derived). While fibroblasts have traditionally been the preferred cellular source for obtaining stem cells, considering the invasive nature of skin biopsy, there is a growing inclination towards more easily accessible cells that do not require invasive procedures for collection. Given these considerations, keratinocytes, urine cells, and blood cells represent valid alternatives [36,37].

Over the years, there has been a development in cellular reprogramming techniques. The initial studies on iPSCs used retroviral or lentiviral vectors, which, by integrating into the cell genome, manipulated gene expression. However, this procedure often led to genomic alterations with aberrant results. Therefore, new transfer systems have been introduced that do not require the integration of foreign gene sequences into the cell DNA. Through the use of non-integrating vectors (such as the Sendai virus or episomal vectors), self-eliminating vectors, and non-viral vectors (such as small bioactive molecules, microRNAs, conventional plasmids, recombinant proteins), it is now possible to reprogram cells without irreversibly modifying their genetic heritage [37–41].

iPSCs can be cultured in feeder-free systems using specialized culture media and extracellular matrix substrates. However, to prevent spontaneous differentiation and maintain pluripotency, a defined culture medium is essential. This medium is characterized by the precise composition of specific chemical components, including growth factors and inhibitor agents. To ensure the quality of iPSC lines, pluripotency markers such as Oct4, Nanog, and SSEA-4 are often detected. Additionally, *in vivo* assessments involve inducing teratoma formation in mice to confirm the iPSCs' ability to differentiate into cells of the three germ layers. To guarantee the stability of the iPSCs' genome, karyotype analysis is conducted. This analysis serves to demonstrate the chromosomal integrity and overall genomic stability of the iPSC lines. These combined measures contribute to the robust maintenance and standardized characterization of iPSCs for research and therapeutic purposes.

Enhanced physiologically relevant 3D models could potentially mimic and present characteristics that are more closely and physiologically related to the human disease process [42]. Concerning animal models, both ethical and technical reasons have confined developmental biology research to selected animal models, which include chicken, frog, zebrafish and mice [42]. This animal studies have all greatly contributed to enhancing the understanding of biological processes and mechanisms. Nevertheless, transcriptional factors and epigenetic regulators function differently in human development. Furthermore, the human genome contains numerous non-coding regulatory elements that could be essential for human embryos and structures to develop and differentiate [42]. For these reasons, a suitable model could include hPSC-derived 3D cultures. These cells can self-organize in cell aggregates that can undergo developmental stages to generate the pseudo-organ of interest (i.e., the spinal cord) [43]. Moreover, spinal cords organoids assessed with this research models could be a key element to help uncover the disease mechanism that could remain otherwise unnoticed and/or not reproducible in 2D models [43].

4. Use of hiPSC in ALS Disease Models

ALS represents the perfect paradigm for using induced pluripotent stem cells (hiPSCs) as disease modelling. In fact, hiPSC, retain the genetic background of donor cells and they can differentiate into different cell types. The latter feature is crucial, since multiple cell types are related to the pathogenesis and the progression of ALS, such as MNs, astrocytes, oligodendrocytes and microglia.

Different studies have investigated transcriptomic ALS signatures in human iPSC models and profiled cultures in bulk [44] or transgenically labeled motor neurons [45]. These studies were performed when neurite degeneration and other phenotypes of overt disease emerged. It is, thus, not possible, in these models, to distinguish early transcriptomics events from secondary transcriptomic events. In trying to overcome those limitations, Ho and colleagues [46] developed a methodology using single-cell RNA sequencing analysis in motor neurons from healthy, familial ALS, sporadic ALS, and genome-edited iPSC lines. These were obtained from multiple patients, batches, and platforms, accounting for genetic and experimental variability to identifying unified and reproducible ALS signatures. The study by Ho *et al*. [46] reported that, by 18 days, iPSC-derived cells showed transcriptional states that globally resemble fetal hindbrain and spinal cord tissue at Carnegie stage 17, or about 42 days of *in vivo* development.

4.1 Differentiation of hiPSCs in MNs

It is well-established that stem cells can differentiate into motor neurons with a high degree of purity (iPSC-derived MNs can be cultured with high purity, induced by small molecules or transcription factors). By using signaling molecules and transcription factors, it is possible to induce the formation of motor neuron subtypes, providing an opportunity to delve into the mechanisms of cellular growth and maturation, as well as any functional alterations that occur during the pathogenetic process.

In fact, multiple studies have demonstrated the feasibility of obtaining hiPSCs from patients with ALS [47]. It has been observed that hiPSC-derived motor neurons (hiPSCs-MNs) maintain physiological characteristics similar to those of endogenous spinal motor neurons or motor neurons derived from embryonic stem cells (ESCs-MNs) [48–51]. Recently, a new method for evaluating cellular physiology called HD-MEAs (high-density microelectrode arrays) has been validated. This method employs microelectrodes to study the functionality of both singular cell and neuronal networks. Results obtained from this new technique confirm that hiPSCs-MNs faithfully reproduce the characteristics of motor neurons, similar to ESCs-MNs and endogenous spinal motor neurons. Therefore, they can serve as an excellent source for studying motor neuron diseases [52]

Over the past decades, researchers have focused on refining protocols for the differentiation of stem cells into motor neurons and currently, there are multiple scientifically accepted methods. The differentiation process varies depending on the type of motor neuron one aims to generate.

The direct differentiation of human induced pluripotent stem cells (hiPSCs) into spinal motor neurons follows a sequential protocol summarized below:

Dual-SMAD inhibition: This involves simultaneously inhibiting the signaling of bone morphogenetic protein (BMP) and transforming growth factor-beta (TGFb) through a process known as dual-SMAD inhibition [53].

Rostral differentiation: Neural progenitors are directed through caudalizing signals, migrating posteriorly in response to caudalizing signals, specifically retinoic acid (RA) for brachial levels and WNT/FGF signaling for thoracic and lumbar levels.

Ventral differentiation: Spinal progenitor cells under the Sonic Hedgehog (SHH) pathway are induced to move anteriorly in response to SHH signaling, inhibiting the expression of dorsal progenitors in a concentration-dependent manner.

Maturation: Neurons are first separated and plated at low density to stimulate neurite outgrowth. The final neural maturation is induced after an interval ranging from 9 to 45 days post-induction. This involves supplementation of the growth medium with brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), or insulin-like growth factor 1 (IGF-1). The first reported generation of hiPSCs and successful differentiation into motor neurons from a patient with Amyotrophic Lateral Sclerosis (ALS) was conducted in the Eggan laboratory, in 2008 [47]. The protocol most commonly used for generating motor neurons from stem cells derived from the study of cells from patients with the C9orf72 mutation. Despite common methodological foundations, the protocols currently employed in various studies exhibit many differences. For example, some avoid retinoic acid signaling, while others use variable concentrations of molecules to induce signaling pathways.

Induced pluripotent stem cells (hiPSCs) can differentiate also in cortical motoneurons. In this case, they distribute radially to form a neuroepithelium, giving rise to what are termed “neural rosettes” [48]. These cells spontaneously arrange in a ventral position [49]. Subsequently, a portion of forebrain glutamatergic neurons migrates to the dorsal telencephalon without the intervention of morphogens [50].

Differentiation protocols for cortical motor neurons do not allow for the generation of neurons from a specific cortical layer. Instead, they lead to the formation of a mixed pool of neurons belonging to different layers of the cortex. Their application in the study of ALS remains uncertain, as it is known that degeneration is confined to a minority of specific cortical motor neurons capable of influencing the physiology of the motor cortex [51].

Due to the considerable variability in differentiation protocols and the inherent heterogeneity of cultured cell lines, there is a critical need for objective methods to classify motor neuron subtypes and improve the purity of cell populations used in research studies. Postmitotic motor neurons can be discerned by examining specific transcription factors, such as ISL1 and HB9, while mature-phase motor neurons are characterized by the expression of choline acetyltransferase and the vesicular acetylcholine neurotransmitter transporter. To recognize motor neurons in every stage of differentiation process, an alternative approach involves the transduction of fluorescently labeled adenoviruses into neural progenitors. This method allows for the tracking of motor neurons throughout its development steps. Noteworthy, studies by Giacomelli *et al.* [51] have contributed valuable insights into these differentiation processes and the identification of motor neuron subtypes.

With regard to ALS modelling, iPSCs have been used to study mutations in genes related to ALS. Studies by Chen *et al.* [54] demonstrated that iPSCs from patients with *SOD1* gene mutations differentiated into spinal MNs, which showed neurofilament aggregation and neurite degeneration related to abnormality in neurofilament regulation. Another study showed that iPSC-derived $SOD1^{+/A4V}$ MNs exhibited disorders in mitochondria and shorter cell survival [55].

Wainger *et al.* [56], using multielectrode array and patch-clamp recordings, demonstrated that hyperexcitability detected by clinical neurophysiological studies of ALS

patients is recapitulated in induced pluripotent stem cell-derived motor neurons from ALS patients harboring superoxide dismutase 1 (*SOD1*), *C9orf72*, and fused-in-sarcoma (*FUS*) mutations. ALS patient-derived motor neurons showed reduced delayed-rectifier potassium current amplitudes compared to control-derived motor neurons. This deficit may sustain their hyperexcitability. Retigabine, a Kv7 channel activator, is able to block the hyperexcitability and improve motor neuron survival *in vitro* when tested in *SOD1* mutant ALS cases [56]. Therefore, electrophysiological characterization of human stem cell-derived neurons may be useful to understand disease-related mechanisms and identify potential therapies.

The pathophysiology assessment of particular types of familiar ALS, however, can represents a limitation for these models, and partially explain why drugs that are promising in preclinical studies fail in clinical trials. Therefore, it is important to focus on a representative model of sporadic ALS, which affects the majority of patients [44].

For this reason, efforts have been made to reprogram sporadic ALS patients' fibroblasts into iPSCs, which can differentiate into neurons with a disease phenotype. These conditions can help represent cellular models to study pathological mechanisms and enhance drug discovery. A study by Burkhardt *et al*. [57] demonstrated that MNs derived from 303 sALS patients showed TDP-43 aggregation.

4.2 Differentiation of hiPSCs in Non-neuronal Cells

It is well known that astrocytes and glial cells play a key role in the pathogenesis of ALS: through a paracrine mechanism, they are capable of creating a pro-inflammatory and neurotoxic microenvironment that triggers neuronal damage and accelerates cellular degeneration. Microglia, as immune cells in the central nervous system (CNS), are important for tissue trophism, maintaining plasticity, and CNS homeostasis. Recent studies indicate that malfunctioning microglia can influence the deposition of neurofilaments in ALS iPSCs-MNs [58]. The use of hiPSC models is crucial for understanding the interactions between stromal cells and MNs in the pathophysiology of ALS. Through these models, it is possible to identify new therapeutic strategies targeting stromal cells with the aim of reducing and modulating neuroinflammation [59].

Differentiation protocols for hiPSCs into astrocytes and glial cells have been developed more recently. Their goal is to generate cells carrying known disease-causing genetic mutations to understand their characteristics and study *in vitro* the complex interactions between non-neuronal cells and human MNs [60]. Abud and colleagues described a protocol to generate human microglia-like cells (iMGLs) from iPSCs. iMGLs resulted to be similar to cultured human adult and fetal microglia, as shown by transcriptomic and functional analyses [61]. They also performed functional assays that emphasized how iMGLs can be used to study microglia genes involved in neurodegenerative diseases both *in vitro* and *in vivo*, by transplanting iMGLs in ALS mouse models. The authors cultured iMGLs with hiPSC 3D brain-organoids (BORGs) to investigate the interactions within a human brain environment. BORGs include different CNS cells such as neurons, astrocytes and oligodendrocytes, organized into a cortical-like network, only microglial cells are lacking [61]. The study showed that, after adding iMGLs to BORG cultures, iMGLs integrated within the 3-D CNS environments and were able to mature, ramify and respond to neuronal injuries as expected by brain microglia. In this way, it is possible to assess the role of microglia in synaptic plasticity, neurogenesis, homeostasis and immune activity. Vahsen and colleagues established a co-culture of iPSC-derived microglia and MNs, allowing the study of MN-micro- glia crosstalk, with particular relevance for ALS research [62]. Co-cultured microglia expressed key ALS-associated genes and released disease-relevant biomarkers. The study showed that different key ALS-associated genes, notably *C9ORF72*, were expressed in the microglia in both monoculture and co-culture models [62]. Furthermore, they found candidate ALS biomarkers to be highly expressed in the microglial cells, including *CHIT1* and *CHI3L* [62].

The process of obtaining astrocytes from hiPSCs (induced pluripotent stem cells) undergoes four main phases in *in vitro* models:

Neural SMAD2 Induction: during this phase, hiPSCs undergo a loss of pluripotency due to the downregulation of Nanog and Oct4 genes, accompanied by the expression of neuronal genes such as Pax6 and nestin. Cells undergo a morphological and functional transformation, organizing into neuroepithelial "rosettes". The addition of growth factors like fibroblast growth factor 2 (FGF-2) or epidermal growth factor (EGF) stimulates the proliferation of these cells.

Addressing cells to Specific Regions: *in vitro*, neural progenitors have a tendency to spontaneously migrate in the telencephalic region. By utilizing morphogenetic factors, cell localization is manipulated both rostro-caudally, through FGF and retinoic acid (RA), and dorso-ventrally through Wnts, bone morphogenetic proteins (BMPs), and sonic hedgehog (Shh).

Gliogenic Switch: in this phase Notch signaling inhibits proneural factors, such as basic helix-loop-helix (bHLH), and promotes astrogliogenesis by activating the JAK-STAT pathway. Other cytokines of IL6 family secreted by the microenvironment, such as leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophin 1 (CT1), converge on the JAK-STAT pathway and contribute to astrocyte development. The interaction between BMPs and these cytokines results in the Smad1-STAT3 complex, which transactivates genes associated with astrocytes. Nuclear factor I (NFI) genes, particularly NFIA, regulate the expression of astrocytic genes GFAP and GLAST. Epigenetic modifications facilitate the

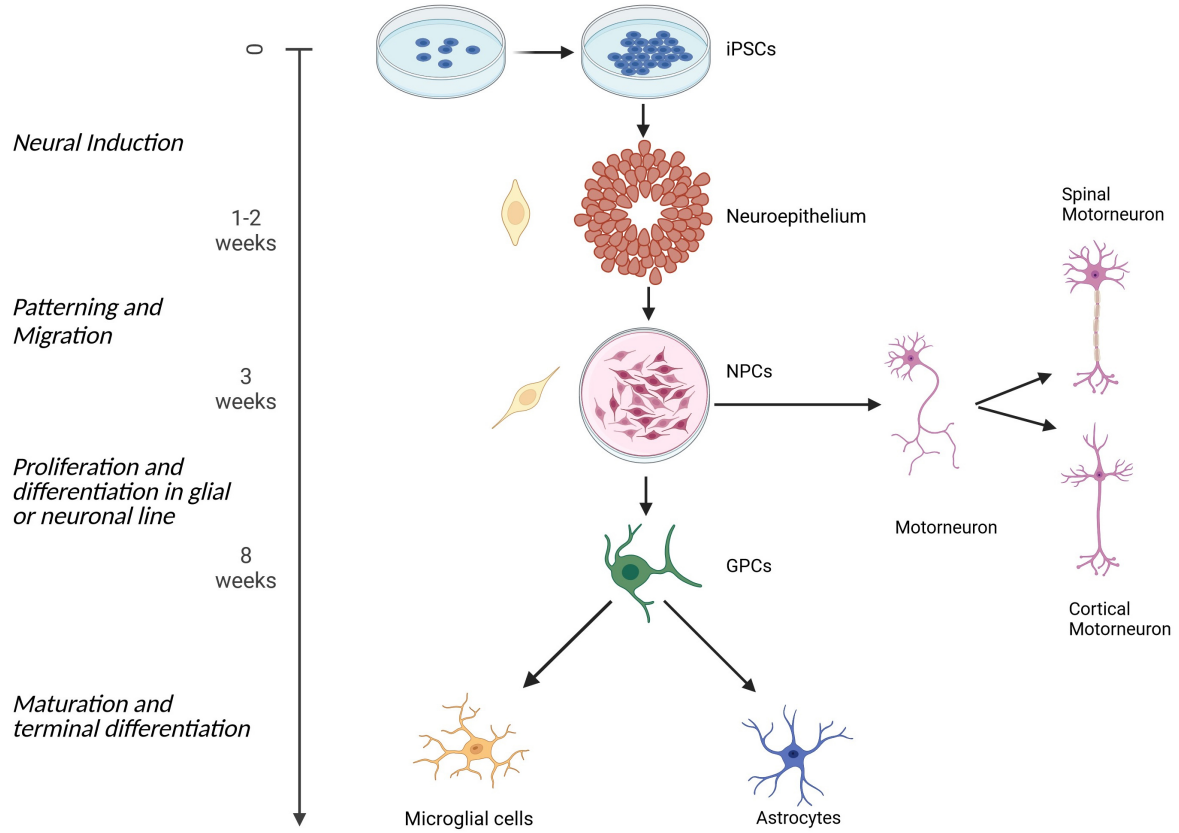

Fig. 1. Differentiation of hiPSCSs in motoneurons and glial cells. iPSCs, Induced Pluripotent Stem cells; NPCs, Neural Progenitors Cells; GPCs, Glial Progenitors Cells (Fig. 1 was created with BioRender.com).

shift of neural precursors towards a glial fate, in particular the demethylation of genes expressed by astrocytes and the diffuse DNA hypomethylation that permits JAK-STAT pathway activation.

Differentiation into Mature Astrocytes: the differentiation into mature astrocytes occurs spontaneously over approximately 120–180 days by withdrawing mitogenic factors from the culture medium. Alternatively, the process can be accelerated making defined culture medium, adding FGF and/or EGF: the gliogenic switch oh NPCs occurs in 12–15 weeks and the immature astrocyte phenotype is confirmed by the expression of markers like NFIA, S100β and CD44. A mild expression of glutamate transporter GLT-1 (known as EAAT-2 in humans) make these cells competent to synaptogenesis process (Fig. 1).

However, *in vitro* models still have many limitations: astrocytes currently derived from hiPSCs are phenotypically immature compared to adult astrocytes and form mixed cellular populations. Effective methods to guide astrocyte differentiation and reproduce region-specific cellular profiles present in the human cortex and spinal cord have not been developed yet. For these cell subtypes, there is still a need to develop protocols that allow better morphological and functional characterization of generated cells, to precisely understand their pathogenic contribution and activity in different areas of the CNS [63].

4.3 hiPSC-derived Models of the Blood-brain Barrier

The blood-brain barrier (BBB) is composed of specialized brain microvascular endothelial cells (BMECs) held together by tight junctions and other supporting cells of the neurovascular unit (NVU) such as pericytes, astrocytes, and neurons [64].

BBB is an extremely selective barrier, essential to maintain brain nutrients homeostasis and to protect the CNS from potentially harmful compounds in the blood (for instance neurotoxins, microbes, etc.). BBB also prevents or limits the penetration of drugs into the brain, being responsible for pharmaco-resistance, as in case of brain tumors, infections and epilepsy.

P-glycoprotein (P-gp), a major multidrug efflux transporter protein expressed at the blood side of the microcapillary endothelium, is an important molecular determinant of the BBB. P-gp acts as an efflux pump that extrudes its substrates into the blood stream, preventing a therapeutic concentration of different drugs from being reached into the CNS [64].

P-gp and other so-called multi-drug resistant proteins are species-specific, thus limiting the utility of animal models in preclinical studies [64].

Table 1. Trials of anti-ALS candidate drugs.

Study	Methods	Drug identified	Mode of action	Effects	Trials
Fujimori *et al*. (2018) [44]	Case cluster model of hiPSCs derived from both FUS-TDP43 fALS and sALS patients	Ropinirole	Agonist of Dopamine receptor (previously approved for Parkinson disease)	- Reduces neurite retraction and cell death, - Anti-oxidative - Inhibition of TDP-43 and FUS depositions - Improvement of mitochondrial activity	ROPALS
Wainger *et al*. (2014) [56]	Electrophysiological experiments on iPSC lines derived from patients with FUS, TDP43 and SOD1 mutations to demonstrate MNS membrane hyperexcitability in ALS disease	Retigabine (ezogabine)	A potassium Kv7 channel activator (previously used as antiseizure drug)	- Lowers cellular hyperexcitability - Contrasts MNs degeneration and death	RTC with ezogabine
Imamura *et al*. (2017) [68]	iPSCs-MNs from ALS patients with *SOD 1* mutations, tested over 1400 compounds and their effects against neurodegeneration	Bosutinib	Inhibitor of the proto-oncogene non-receptor protein tyrosine kinase (Src/c-Abl)	- Promotes autophagy - Reduces SOD1 misfolded proteins aggregates - Reduces cell death	iDReAM
Tsuburaya *et al*. (2018) [70]	160.000 compounds tested on ALS models with SOD1 mutation, *in vitro* using MNs derived from iPSCs and *in vivo* using mice	Compound 56	Inhibitor of SOD1- Derlin 1 interaction, trigger of motoneuron death	- Prolongs survival in animal models (mice): slow progression and late onset of disease	Preclinical phase
Kato *et al*. (2021) [71]	Fluorescence-labeled TDP43 gene was transduced in hiPSCs and differentiated in MNs, in order to test drugs effects on TDP43 mislocalization	Niclosamide	Inhibitor of STAT3 pathway	- Prevent TDP43 misclocation and degradation - Reduces morphological changes	None
Kuta *et al*. (2020) [72]	Testing stress response inducing drugs on iPSCs derived spinal motor neurons carrying the FUS p525L mutation	SAHA, RGFP109 and Arimoclomol	Inhibitor of histone deacetylase HDAC, upregulation of heat shock proteins response	- Reduce loss of nuclear FUS - Restore DNA repair activity (associated to Arimoclomol)	None

hiPSCs, Human Induced Pluripotent Stem Cells; FUS, Fused in Sarcoma; TDP43, TAR-DNA binding protein-43; fALS, familial Amyotrophic Lateral Sclerosis; sALS, sporadic Amyotrophic Lateral Sclerosis; ROPALS, Ropinirole hydrochloride remedy for amyotrophic lateral sclerosis; iPSC, induced Pluripotent Stem Cells; SOD1, superoxide dismutase 1; MNs, Motor Neurons; ALS, Amyotrophic Lateral Sclerosis; iDReAM, Induced pluripotent stem cell-based Drug Repurposing for Amyotrophic lateral sclerosis Medicine; HDAC, Histone Deacetylase; RTC, Randomized Controlled Trial; HSP, Heat Shock Protein.

In recent years, the increased understanding of the molecular signaling events that occurring during the prenatal development of BBB, allows considerable improvements in differentiating BMECs from iPSCs [65].

iPSC- derived BMECs (iBMECs) have advanced *in vitro* modeling of the human BBB, increasing our knowledge of human BBB development and function as well as facilitating CNS drug studies [65].

Barrier breakdown and dysfunction have been observed in all major neurodegenerative diseases and likely contributes to the pathogenesis of many neurological disorders [66].

The application of iPSC-derived BBB models helped to understanding BBB dysfunction and studying disease mechanisms in a tailored manner. This approach also offers the opportunity to investigate the earliest stages of BBB breakdown, which can be difficult to ascertain from *post-mortem* tissues.

BBB dysfunction has also been found in several iPSC-derived BBB models of the most common neurodegenerative diseases. Using iPSCs from patients with familial forms of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and ALS, Katt and colleagues [66] demonstrated that these patient- derived iBMECs had different kinds of BBB impairment.

Interestingly, in ALS iPSC-derived astrocytes from SOD1 and sporadic patients, an upregulation of P-gp has been observed in co- cultured iBMECs [64,67]. The upregulation of this efflux transporter may limit the delivery of therapeutic drugs to the CNS.

Continued development of iPSC-derived BBB models may lead to improvements in the understanding of human BBB functions and the pathogenesis of neurological diseases, allowing the development of novel BBB-permeable drugs to target the CNS.

5. Therapeutical Implications

Stem cell technology has significant applications in therapeutic contexts, enabling the development of new therapeutic approaches and providing models for testing the efficacy and safety of emerging drugs.

5.1 Drug Screening

iPSC-derived motor neurons from patients with both sporadic ALS and types of ALS carrying mutations in genes including *SOD1*, *TDP-43*, *FUS*, and *C9orf72* have been used in drug screening studies [44,56,68–72].

Interestingly, three candidate anti-ALS drugs, ropinirole (ROPI), retigabine, and bosutinib, have been identified in iPSC-based drug screenings (Table 1, Ref. [44,56,68,70–72]).

ROPI (Ropinirole) is categorized as a dopamine receptor agonist commonly used in Parkinson Disease. As possible therapy for ALS, it was discovered through a drug screening analysis conducted at Keio University on motor neurons derived from iPSCs carriers of FUS and TDP-43 mutations [73]. This drug showed an antiapoptotic effect in iPSC-derived spinal MNs from both sporadic and familial ALS patients' tissues [73]. Ropinirole has been shown to improve mitochondrial activity by inhibiting TDP-43 and FUS aggregation, and reducing oxidative stress. These activities may reduce the retraction of neurites and the loss of neurons in ALS patients with FUS and TDP-43 mutations, as well as in some cases of sporadic ALS. Currently undergoing testing in the ROPALS trial [74].

Retigabine is as an activator of voltage-gated potassium channels (Kv7), previously approved as anti-seizure medication, but early abandoned due to irreversible side effects. From electrophysiological analysis conducted on motor neurons derived from ALS iPSCs effect on modulating the hyperexcitability status in these cells has been observed. A Phase II Pharmacodynamic Trial of Retigabine in ALS patients was realized from 2015 to 2019 and demonstrated a reduction in excitability of cortical and spinal motor neurons in ALS participants. However, it is important to note that the study was not designed to reveal an impact on the natural course of the disease [75].

Bosutinib is an inhibitor of the proto-oncogene nonreceptor protein tyrosine kinase (Src/c-Abl), actually still under further investigation trough a phase I clinical trial started in March 2019. Its pharmacodynamics is based on the ability to hinder the aggregation of misfolded Superoxide Dismutase 1 (SOD1), preventing cell death in cases of both genetic and sporadic ALS [69].

With the development of organoid technology, extensive 3D model systems can be obtained to study ALS and optimize drug screening and discovery [76]. A recent study described a 3D organoid model of ALS from both human iPSC-derived muscle fiber cells and human ESC-derived MNs obtained from a patient with ALS harboring *TDP-43* mutation [77]. In another study, ALS organoids treated with rapamycin and bosutinib presented improved muscle contraction and motor neuron viability when compared to untreated organoids. These results support the possible use of the organoids for candidate drugs screening and prediction of response to treatments [78].

5.2 Replacement Therapy

Induced pluripotent stem cells (iPSCs) represent an inexhaustible source of nerve cells, and they are employed in innovative therapies, such as tissue regeneration. In addition to investigations uncovering molecular therapeutic targets and elucidating critical pathogenetic mechanisms in ALS, there has been a concerted exploration about transplantation of iPSC-derived motor neurons (MNs) into the central nervous system (CNS) of patients with ALS. Even if it is still under investigation, recent evidences by preclinical models suggest that the potential of this treatment lies more in the ability of transplanted cells to reconstruct

a protective microenvironment for motor neurons, capable of counteracting degeneration and slowing cell loss. iPSC-NSCs (neural stem cells derived from induced pluripotent stem cells) can differentiate into glial and neuronal cells and exert neurotrophic, anti-inflammatory, and immunomodulatory effects through a paracrine mechanism [79].

Sareen *et al*. [80] successfully generated neural progenitor cells (NPCs) displaying a spinal cord phenotype from iPSCs and transplanted them into the spinal cords of murine models. The study demonstrated the survival of these cells *in vivo* and the capacity of this cells to differentiate into glial cells under the influence of the host microenvironment [80].

Concurrently, Kondo *et al*. [81] confirmed the pathogenetic significance of glial cells in ALS and explored their potential therapeutic utility by transplanting glial precursor cells derived from stem cells. When introduced into mice with ALS, these cells not only retained the capability to mature into astrocytes but also enhanced the survival of motor neurons by establishing a supportive microenvironment [81].

Several experimental studies conducted over the past decade have indicated that *in vitro*-derived neural precursors, when transplanted *in vivo*, can integrate into host tissue and differentiate into both glial and neuronal cells. This process exerts therapeutic effects through immunomodulation and the release of growth factors. For instance, Nizzardo *et al*. [82] administered neural precursors obtained from human induced pluripotent stem cells via intrathecal or intravenous injections in ALS-afflicted mice, resulting in a clinical improvement in neuromuscular functions. Similarly, these researchers performed a study on *SOD1G93A* transgenic rats, treating them with intraspinal stem cell injections [83]. In these studies, the outcomes were promising, resulting in an improvement in disease phenotype and a favorable prognosis.

However, the curative effectiveness of directly replacing dead neurons with stem cells and their application on clinical studies have not been demonstrated yet. To regenerate lost tissue, transplanted motor neurons would need to continue dividing and form axons directed at a distance to create a functional synapse with the muscle target. Currently, achieving these results *in vivo* is not possible. Furthermore, this field of research still faces numerous economic and ethical obstacles [84,85].

5.3 Gene Therapy

Research on gene therapies is still in the process of refinement, and the use of stem cells in this field has yielded many results. In fact, on *in vitro* models, it is possible to identify therapeutic targets and directly observe the phenotypic consequences of genetic manipulations performed on cells [86]. An example of gene therapy in ALS is linked to the use of antisense oligonucleotides (ASOs) for correcting genetic mutations widely recognized as causative of the disease. ASOs are synthetic chains of nucleic acids that can bind to specific RNA sequences and modulate transcription and gene expression of the cells [87]. Techniques aimed at improving the stability of these molecules and their binding affinity with target sequence are still under investigation, with the goal of maximizing their bioavailability and minimizing possible side effects resulting from their use.

6. Conclusions

In conclusion, in this review, we aim to describing the potential of hiPSC (induced pluripotent stem cell) technology, which enables the generation of infinite and renewable pools of ALS (Amyotrophic Lateral Sclerosis)-relevant cell types. The genetic heritage of a given patient is preserved during cell reprogramming *in vitro*, and this allows to reproduce the inter-individual heterogeneity and to study the numerous disease-causing mutations and the complexity of interactions between different cell subtypes.

New differentiation protocols have recently been introduced, and cell reprogramming technology continues to evolve. Through *in vitro* models, the key role of non-neuronal cells in ensuring the proper functionality of neuromuscular tissue has been discovered. All these elements contribute to making the use of stem cells a real diagnostic and therapeutic hope for motor neuron diseases in the not-too-distant future. However, there are still many challenges to overcome in order to achieve satisfactory results and to transfer the models *in vivo*. It is necessary to standardize cell differentiation technology to make scientific evidence more concrete, replicate the signs of cellular aging typical of neurodegenerative diseases that are lost during reprogramming into pluripotent cells, and finally, expand the data on ALS phenotypes, especially for sporadic forms, which are currently less described compared to familial forms.

One of the critical problems encountered in using patient-specific iPSC-derived from clinically relevant phenotypic cells as *in vitro* disease models is represented by the fact that the majority of these iPSC-derived phenotypic cells exhibit immature functional characteristics. These features are similar to their respective embryonic or fetal phenotypic cells. Moreover, they also contain a heterogenous mixture of varying proportions of phenotypic subtypes, giving rise to a variety of differentiations [33].

The development of a reliable disease model with defined cellular characteristics and a homogenous population of the phenotypic cells of interest is a crucial pre-requisite for developing therapeutic applications [33].

iPSC-based disease modelling has been widely used for early-onset diseases and proved to be successful model for this kind of diseases, such as spinal muscular atrophy.

For late-onset conditions, the disease models are prone to fail considering that the iPSC-derived relevant phenotypic cells lack the adult maturation characteristics to exhibit the disease phenotype [33].

There have been several approaches to induce the maturation of these primitive iPSC phenotypic cells, which include treatment with mitochondrial stress inducers and inhibitors of protein degradation, 3D co-culture to enhance paracrine-mediated stimulation of ageing and maturation, improved formulation of cultured/conditioned medium and derivation of iPSCs from adult aged patients [33]. Cell maturation obtained tends to be modest, despite all these efforts.

An alternative approach could be the direct conversion of somatic cells such as fibroblasts into clinically relevant phenotypic cells, to preserve the cellular ageing of markers.

Availability of Data and Materials

Data and Materials pertinent to the study are all provided in the manuscript.

Author Contributions

LC, LV and GP designed the study, wrote the manuscript editied the paper and approved the final version; MV, TI, CS, LS and MZ assisted in the research, editing, preparation and approval of the final version of the article. All the authors contributed to editorial changes in the manuscript. All the authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

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Conflict of Interest

The authors declare no conflict of interest.

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