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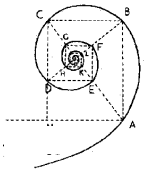

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TRANSPLANTATION OF A SPECIFIC NEURAL STEM CELL SUBPOPULATION AS A THERAPEUTIC APPROACH FOR AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a fatal neuromuscular disorder caused by degeneration of motor neurons (MNs) in the spinal cord, brainstem, and cortex. It belongs to a group of heterogeneous disorders called "motor neuron diseases", in which ALS is the most common form in adults. The progressive MN degeneration leads to a gradual muscle atrophy and paralysis. Patients affected by ALS usually die 3-5 years after the onset of symptoms due to respiratory failure. Up to now, no effective cure is available for ALS beyond supportive care and Riluzole, which only modestly prolongs survival.

In the early stage of the disease, MN loss and consequent muscle denervation are compensated by axonal sprouting and reinnervation by the remaining MNs, but this mechanism is insufficient in the long term.

Thanks to their multiple beneficial mechanisms, stem cell transplantation represents a promising therapeutic strategy for ALS and other neurodegenerative disorders. In fact, transplanted stem cells can provide therapeutic effect by modulating the micro-environment through the production of neurotrophic factors, eliminating toxic molecules, reducing neuroinflammation and generating auxiliary neural networks. Moreover, stem cells can eventually replace degenerating cells. A novel source for stem cell transplantation consists in the reprogramming of adult somatic cells into induced pluripotent stem cells (iPSCs). Since iPSCs are directly derived from adult tissues, they bypass ethical issue of the embryo manipulation and are patient-specific, potentially reproducing ALS features in vitro. This means that they are a promising tool for stem cells transplantation and to model human pathologies in vitro.

In this study, we isolated a specific subpopulation of neural stem cells (NSCs) derived from differentiated iPSCs. Compared to other types of stem cell, NSCs are particularly appropriate for ALS treatment due to their peculiar ability to differentiate into neurons, astrocytes and oligodendrocytes.

The rationale of this study consists in the selection of a subpopulation of NSCs able to engraft and migrate through the nervous system parenchyma, to protect degenerating MNs and to improve ALS phenotype.

We selected NSCs for the presence of three markers: Lewis X (or LeX), CXCR4 e β1 integrin. Lewis X is a glycoprotein marker of stem cells with a relevant role in cell adhesion and migration. CXCR4 is a chemokine receptor, which increases the sensitivity of the cells to be recruited by the host spinal cord that produces chemoattractant cytokines. β1 integrin is a subunit of VLA4, a receptor that allows cells to cross the blood-brain barrier, particularly in the presence of inflammation as in ALS animal models and human patients. In order to evaluate the ability of LeX+CXCR4+β1+ NSCs to engraft into the nervous system and to improve ALS phenotype, we performed intrathecal injection of these cells in the SOD1G93A mouse model.

Transplantation resulted in an efficient engraftment of the cells, which reached central nervous system bypassing blood brain barrier, and in the protection of MNs and their axons from degeneration.
This determined a preservation of neuromuscular junction (NMJ) innervations by maintaining their integrity and inducing axonal sprouting. These beneficial effects on neuropathological phenotype correlated with a significant increased survival and improved neuromuscular function of transplanted SOD1G93A mice.
We also demonstrated the beneficial effects of LeX+CXCR4+β1+ NSCs in a human in vitro model of ALS. When co-cultured with these cells, iPSC-derived MNs from ALS patients showed an improvement in terms of survival and axonal growth.
We then analyzed the molecular mechanisms underlying NSC protection demonstrating that our NSC subpopulation exerted positive effects through neurotrophic factors production, inhibition of the GSK3β activity, and limiting astrocytes proliferation through activation of vanilloid receptor.
The results of this study suggest that effective protection of MNs and NMJs can be achieved targeting multiple deregulated cellular and molecular mechanisms in both MNs and glial cells in ALS models.
This is particularly relevant for ALS because different pathological mechanisms likely contribute to its onset, making NSC transplantation a promising therapeutic approach for ALS.

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ABBREVIATIONS

ALDH = aldehyde dehydrogenase

ALS = amyotrophic lateral sclerosis

ALSFRS-R = amyotrophic lateral sclerosis functional rating scale-revised

ASO = antisense oligonucleotide

BBB = blood-brain barrier

BCL2 = b-cell leukemia/lymphoma 2

BDNF = brain-derived neurotrophic factor

BMP = bone morphogenic protein

ChAT = choline acetyltransferase

CNS = central nervous system

CSF = cerebrospinal fluid

CXCR4 = chemochine receptor 4

CZP = capsazepine

DPR = dipeptide repeat proteins

EB = embryoid body

EEAT2 = excitatory amino acid transporter 2

ELISA = enzyme-linked immunosorbent assay

ER = endoplasmic reticulum

ESC = embryonic stem cell

FACS = fluorescence activated cell sorting

fALS = familial amyotrophic lateral sclerosis

FGF = fibroblast growth factor

FTD = frontotemporal dementia

FUS = fused in sarcoma

GDNF = glial cell-derived growth factor

GFAP = glial fibrillary acid protein

GFP = green fluorescent protein

GSK-3PSe9 = glycogen synthase kinase 3 beta phosphorylated in serine 9

GSK3β = glycogen synthase kinase 3 beta

GST = grip strength testing

Hb9 = homeobox 9

HHD = hand-held dynamometry

hnRNP = heterogeneous nuclear ribonucleoprotein

HSTF-1 = heat shock transcription factor 1

IGF = insulin-like growth factor

iPSC = induced pluripotent stem cell

iPSC-derived MNs = induced pluripotent stem cells-derived motor neurons

I-RTX = iodoresiniferatoxin

KLF4 = krueppel-like factor 4

LeX = lewis X (or CD15)

MACS = magnetic-activated cell sorting

MAP2 = microtubule-associated protein 2

MN = motor neuron

NMJ = neural muscular junctions

NSC = neural stem cell

NT3 = neurotrophin-3

OCT4 = octamer-binding transcription factor 4

OriP/EBNA1 = OriP/Epstein–Barr nuclear antigen 1

PAX6 = paired box homeotic gene 6

PI3K = phosphoinositide 3 kinase

PLL = poly-L-lysine

RA = retinoic acid

RAN = repeat-associated non-ATG

sALS = sporadic amyotrophic lateral sclerosis

SD = standard deviation

SDF1 = stromal cell-derived factor 1

SEM = standard error of the mean

SHH = sonic hedgehog

SOD1 = superoxide dismutase 1

SOX2 = sex determining region Y-box 2

SSEA = stage-specific embryonic antigen

TARDBP = TAR DNA-binding protein 43

TGF = transforming growth factor

TNF = tumor necrosis factor

TRA = tumor rejection antigen

TRPV1 = transient receptor potential vanilloid subfamily member 1

VCAM = vascular cell adhesion protein

VEGF = vascular endothelial growth factor

VLA4 = very late antigen 4

WT = wild type

ZFN = zinc finger nuclease

1. INTRODUCTION

1.1 Amyotrophic Lateral Sclerosis

1.1.1 Clinical features

Amyotrophic Lateral Sclerosis (ALS) is a rapidly progressive and invariably fatal neuromuscular disorder. Also known as Lou Gehrig's disease for the famous baseball player who died of this disorder, ALS is characterized by gradual degeneration and death of motor neurons (MNs), the nervous cells that control links between the nervous system and muscles [1]. In ALS, both upper MNs in the cortex and lower MNs in the spinal cord die, stopping sending messages to muscles. This leads to a progressive muscle weakness, atrophy and paralysis in few years [2] (Fig. 1).

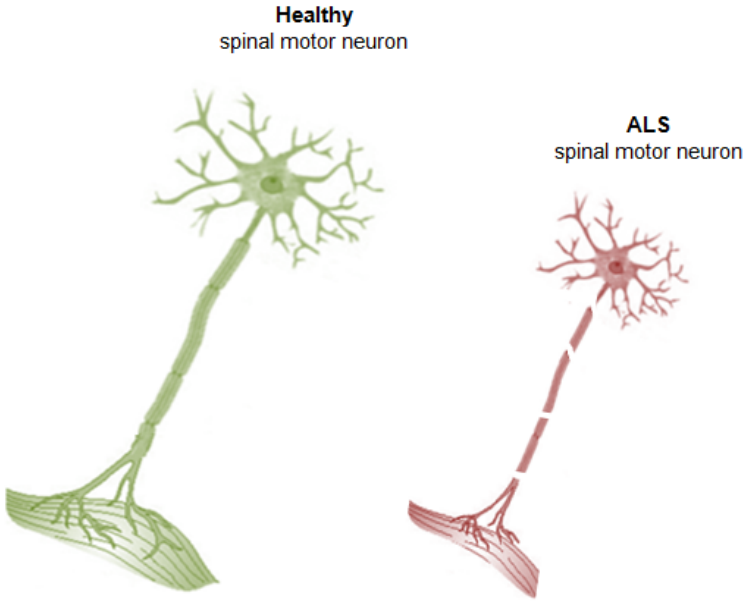

Fig.1 Illustrative comparison between healthy and ALS MNs.
In ALS, spinal MNs are characterized by degenerating nerves, which cannot send message to muscles, causing their atrophy and paralysis.

The ALS belongs to a group of heterogeneous pathologies called “motor neuron diseases”, in which ALS is the most common and severe form in adults [3]. Patients affected by ALS usually die 3-5 years from the onset of symptoms due to respiratory failure [4], and only the 35% of patients survive 5 years or more [5].

The term “Amyotrophic” is referred to the muscle atrophy, whereas “Lateral Sclerosis” is referred to the hardness to palpation of the lateral columns of the spinal cord in autopsy specimens [6]. The typical onset of ALS consists in an asymmetric weakness in a limb that causes unexplained foot drop and falls [6, 7]. Weakness rapidly spreads to contiguous limb regions and finally reaches respiratory muscles [8]. For this reason, ALS patients progressively lose their muscle strength and the ability to move their arms, legs and body. When paralysis reaches the diaphragm and chest wall, patients lose the ability to breathe without ventilatory support.

Although approximately 65% of ALS cases are known as “spinal forms” with the initial involvement of the limbs, often asymmetric, the 30% of cases are “bulbar forms”, in which the ALS starts with dysarthria or dysphagia. Only 5% of ALS cases begin aggressively with early respiratory failure [9, 10]. ALS usually does not affect cognitive functions, even if a subset of ALS patients has also a frontotemporal dementia or a subtle frontal lobe deficit. Also sight, hearing, taste, smell and touch are not compromised in ALS. Until the late stages of the disease, patients usually maintain control of eye muscles and functions of bladder and bowel [11]. The reasons of this relative sparing are unknown. Patients with ALS show a wide combination of symptoms and signs (Fig. 2) due not only to the site of onset, but also to the rate of ALS progression and the relative number of upper and lower MN deficits [8].

As a result of upper and lower MNs degeneration, clinical examination reveals a combination of upper MNs signs such as spasticity, hyperreflexia, hypertonia, extensor plantar response (or Babinski sign) and lower MN signs that include muscle atrophy, weakness, fasciculations and cramps [12].

The propagation of ALS through the nervous system is not fully elucidated. It has been hypothesized that the focal damage of a localized group of MNs spreads to adjacent MNs [13], however is not clear whether ALS onset is primarily in the upper or lower MNs.

The vast majority of ALS cases is sporadic (sALS), with no prior family history, and only a 10% of ALS cases are inherited or familial (fALS), usually as dominant traits [14]. The incidence of ALS is 2.16 per 100.000 person/years [15] and there is no relevant difference between countries [16]. The lifetime risk of ALS is approximately 1:400 for women and 1:350 for men [17]. The peak incidence occurs between 58-63 years for sALS and 47-42 years for fALS, decreasing rapidly after 80 years of age [18].

Currently, there is no cure for ALS beyond supportive care and Riluzole, a putative glutamate release blocker that modestly prolongs survival.

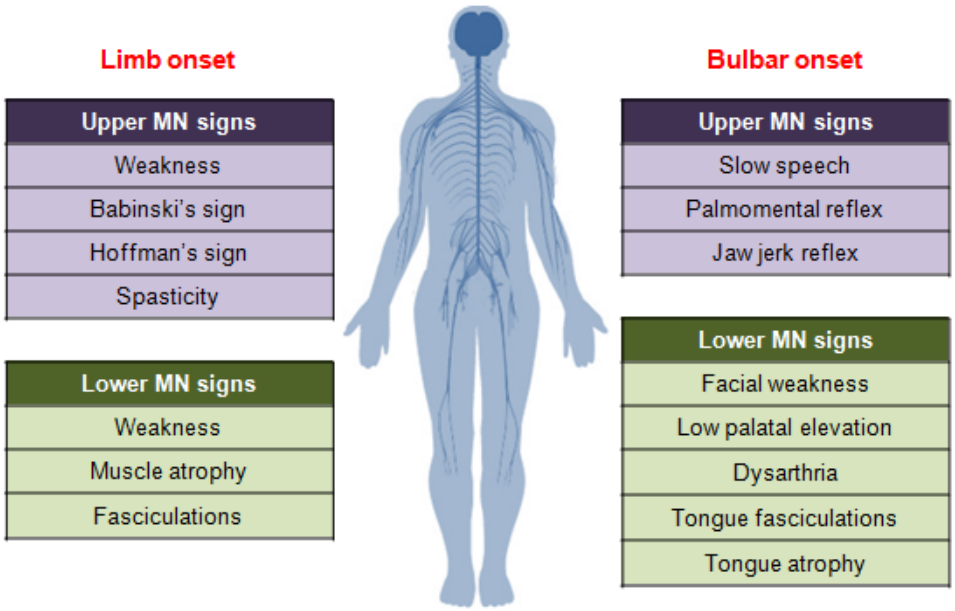

Fig. 2 Schematic representation of ALS symptoms.

Classification of ALS symptoms according to their onset, which could be bulbar or limb, and the type of MNs involved, which could be upper in cortex or lower in spinal cord.

1.1.2 Aetiopathogenesis

Despite several studies aim at elucidating the ALS causes, the aetiopathogenesis is still unclear. In fact, ALS is a complex disorder involving an intricate combination of events including common and rare genetic, environmental and lifestyle factors [17].

The vast majority of ALS cases are sporadic (sALS), without a clear genetic linkage. Only the 10% of cases show familial inheritance, and are defined familial ALS or fALS [19]. However, the boundaries between fALS and sALS cases are undetermined due to the existence of sporadic cases associated with mutations in the same genes of familial ALS patients [5]. Furthermore, because ALS is a disease of the adulthood, the collection of large pedigrees suitable for linkage studies is often difficult, complicating the identification of ALS causative genes.

Genetic causes

The involvement of genetic factors in ALS occurs at various levels and now is identified in up to two-thirds of familial cases and 10% of apparently sporadic ALS. The genes involved in ALS frequently lead to a classic autosomal dominant pattern of inheritance, but can also be recessive or X-linked. Moreover, there are susceptibility genes that might enhance the risk of developing a sporadic form of the disease [5].

The most significant causative genes include:

- SOD1: it was the first causative gene of ALS identified. Discovered in 1993 by Rosen et al., SOD1 gene encodes for copper/zinc ion-binding superoxide dismutase 1, an antioxidant enzyme that protects cells from the toxic effects of free radicals [20]. Alterations in SOD1 are particularly frequent and linked to 20% of ALS cases and 3% of apparently sporadic disease [21]. To date, more than 170 mutations have been reported along the peptide sequence of SOD1, causing a misfolding and aggregation of the mutant protein [22]. The mutant misfolded SOD1 is targeted for degradation through ubiquitylation; however, it escapes this regulatory process in the cell, gaining novel and toxic functions that leads to MN degeneration [23].

Many different toxic mechanisms of mutant SOD1 have been suggested such as excitotoxicity [24], oxidative stress [25], failure of glutamate transporter [26], protein aggregation [27], aberrant protein-protein interactions [28], apoptosis [29], low binding affinity of zinc [30], mitochondrial dysfunction [31] and endoplasmic reticulum stress [32]. Interestingly, recent discoveries reveal that in some sALS patients, changes in oxidation, demetallation and other post-translational modifications occur, inducing aberrant conformations of wild-type (WT) SOD1. This leads to acquisition of toxic functions comparable to those of fALS patients with SOD1 mutations [33]. This finding suggests a role for WT SOD1 in sALS, possibly after oxidative modification.

The phenotype is very similar among patients with mutations in SOD1, even though the onset and progression can be different depending on alternative SOD1 mutations [34].

- C9orf72: recently discovered in 2011 [35, 36], the alteration in C9orf72 gene consists in a hexanucleotide repeat expansion of GGGGCC in a non-coding region of chromosome 9 open reading frame 72 (C9orf72). The C9orf72 repeat expansion represents the most common genetic cause of fALS (30-50%) and it is also reported in a significant percentage of apparently sporadic ALS cases (4–10%) [36, 37]. Moreover, the (GGGGCC)n repeat expansion in C9orf72 determines many familial frontotemporal dementia (FTD) cases (~25 %), explaining the overlap between ALS and FTD [38, 39]. The normal number of GGGGCC repeats in healthy persons is between 2 and 23 repeats, patients with ALS and FTD show a range of 700 to 1600 repeats [35, 36].

The function of the protein encoded by C9orf72 gene is still unknown; however, the molecular mechanisms that underlie C9orf72-associated ALS are rapidly emerging [40]. In particular, three potential pathogenic mechanisms have been postulated: (1) loss of function owing to reduction in the transcript levels of C9orf72 mRNA variants in cells and tissue of ALS patients that may cause defects in autophagic and endosomal processes and MN function [36, 41-44]; (2) gain of RNA toxicity due to sense (GGGGCC) and antisense (GGCCCC) transcripts that accumulate in RNA foci and sequester critical RNA-binding proteins [36, 44-47]; (3) proteotoxicity from 6 dipeptide repeat proteins (DPR) produced by an unconventional form of translation (RAN translation) of the expanded repeat [48-51]. These DPR proteins are generated by translation in all frames of the GGGGCC repeat resulting in polymers rich of glycine-alanine (GA), glycine-proline (GP), glycine-arginine (GR), glycine-proline (GP), alanine-proline (AP) and proline-arginine (PR).

C9orf72-related ALS patients have a wide range of phenotypes and also the age at onset vary between studies [52].

- TARDBP: also known as TDP-43, it encodes for a DNA and RNA binding protein. Its target sequences are mainly intronic and rich in UG sequences, but also include non-coding RNAs and 3′ UTRs [53]. In line with this, TDP-43 plays a role in nuclear RNA metabolism including gene splicing [54], transcription regulation, processing of small regulatory RNAs (microRNAs), mRNA nucleo-cytoplasmic shuttling and RNA transport [55]. To date, the vast majority of mutations in TDP-43 are localized in the C-terminal domain, which is involved in binding other hnRNPs and is important for the splicing activity of TDP-43 [56]. TARDBP mutations have been found in 4-6% of fALS cases without SOD1 mutations and may be responsible for a small percentage (0.2%) of sALS patients and FTD [57].

Following the discovery of SOD1 aggregates, a breakthrough was achieved with the identification of TDP-43 as a major component of ubiquitinated inclusions in FTD and ALS cases [58]. Inclusions of TDP-43 are also found in ALS patients without TDP43 mutations; in fact, non-mutated TDP-43 forms aggregates in all sALS patients and the vast majority fALS SOD1 negative, but not in SOD1 related ALS [59].

It is difficult to establish clear correlation between phenotype and genotype and is clinically impossible to discriminate TDP-43 patients from other ALS patients [16]. The onset of symptoms in TDP-43 patients can vary from 20 to 77 years old [60].

- FUS: it encodes for a RNA binding protein named fused in sarcoma. Like TDP-43, it is involved in RNA splicing, transport and translation, genomic maintenance, and transcription factor regulation. It has also a role in microRNA processing [57]. FUS mutations have been found in 4-6% of fALS and 0.7-1.8 of sALS cases [61]. Mutations in TDP-43 and in FUS, both involved in RNA metabolism, have focused the attention on the roles of this altered process in the development of ALS pathology. However, it remains unclear if the toxicity in mutant FUS cases is due to abnormal RNA modulation and metabolism, or to an altered localization of the protein in the cell [62].

Other genes are associated with a minor proportion of ALS patients (Table 1), while the vast majority of patients diagnosed with ALS are negative for mutations in known genes and up to now are considered as having a sporadic disease.

Table 1. Minor genes involved in ALS

Minor ALS genes	Protein	Function
ANG	Angiogenin	Blood vessels formation
OPTN	Optineurin	Vescicular transport
UBQLN2	Ubiquilin-2	Proteasome
DCTN1	Dynactin 1	Cytoskeleton dynamics
ALS2	Alsin 2	Endocytosis
CHMP2B	Charged Multivesicular Body Protein 2B	Vescicular transport
FIG4	Phosphoinositide 5-phosphatase	Vescicular transport
HNRNP-A1 / A2B1	Heterogeneous nuclear ribonucleoprotein A1 and A2/B1	RNA metabolism
SETX	Senataxin	RNA processing
SPG11	Spastic paraplegia 11	DNA damage
VAPB	Vesicle-associated membrane protein-associated protein B	Vescicular transport
NEFH	Neurofilament heavy	Cytoskeleton dynamics
ARHGEF28	Rho Guanine Nucleotide Exchange Factor 28	RNA metabolism
SQSTM1	Sequestosome 1	Ubiquitination dynamics
VCP	Valosin containing protein	Vescicular transport
PFN1	Profilin 1	Cytoskeleton dynamics
PRPH	Peripherin	Cytoskeleton dynamics
DAO	D-amino acid oxidase	Neurotransmitter metabolism
SIGMAR1	Sigma non-opioid intracellular receptor 1	Calcium metabolism
TAF15	TATA-box binding protein associated factor 15	DNA damage
ERBB4	Erb-2 receptor tyrosine kinase 4	Neural development
MATR3	Matrin 3	Transciption dynamics
TBK1	TANK-binding kinase 1	Apoptotic pathway

Environmental factors

Several environmental factors have been proposed to be linked with ALS, but none of them are clearly established.

Risk factors include pesticide contamination, metals, smoking, alcohol, lower body mass index, viral and fungal infections, head trauma, metabolic and inflammatory states, cancer and electromagnetic fields [63], as well as the exposure to unknown toxins, as detected among soldiers of Persian Gulf war [64]. Some studies have suggested a possible correlation between ALS and physical activity, because ALS incidence is higher in football and soccer players (respectively 40 and 6.5 fold), but this hypothesis has to be confirmed [65]. To date, the only established risk factors are older age, male gender and a family history of ALS [66]. In contrast, hypercholesterolemia, the use of statins and immunosuppressive drugs are associated with a decreased risk of ALS.

1.1.3 Animal models

In order to extricate the entangled underlying pathomechanisms of ALS and test new therapeutics, a consistent number of animal models have been generated starting from knowledge on the genetic causes of ALS.

ALS animal models include zebrafish, dog, drosophila melanogaster, C. elegans, and pig; however, the most used animals in pre-clinical trials are rodents (rats and mice).

The first animal model that has been established for ALS is a transgenic mouse expressing multiple copies of human SOD1 gene with G93A mutation. This model develops a MN disease that recapitulates most of the clinical and neuropathological findings of familial and sporadic ALS [67]. SOD1G93A mice develop ALS symptoms at 80-90 days as shown by rotarod and hang-wire tests and they die at a median age of 140 days. However, it has been demonstrated that the degeneration of the neuromuscular junctions (NMJs) starts before the onset of symptoms, around 40 to 50 days [68]. Also the gliosis was found before onset of ALS and increased in intensity over time [69]. In addition to the original mutant SOD1G93A mice, a large number of other SOD1 transgenic mice have been created that include G37R (the second most used), G85R, D90A, G86R, mice with SOD1 truncated at the C-terminus.

Most mice with SOD1 mutations represent human SOD1-associated ALS quite well. In fact, mice develop fatal paralysis with MN deficit, gliosis and SOD1 aggregates. However, these mice exhibit variable phenotypes, age of onset and survival that seem to be dependent on specific mutations, levels of mutant SOD1, gender and genetic background [16].

Despite a large number of attempts, the rodent models of TDP-43 are not perfect yet and none of these models is currently used for routine drug screening. These animals are characterized by varied phenotypes without a clear correlation between mutations and phenotypes, which is highly dependent upon the promoter used and the level of TDP-43 expression. In fact, low protein expression induces neuromuscular dysfunction and accumulation of TDP-43, but mice did not get paralyzed [70]. Vice versa, high expression of TDP-43 causes early onset and fast disease progression but without clear MN loss. In this case, death seems to be due to gastrointestinal complications rather than neurodegeneration [71]. More recently, a promising TDP43 model is the double transgenic mouse, which expresses human TDP-43 wild type and human TDP-43 with Q331K mutation. This model recapitulates most ALS features in exclusion of age-related degeneration [72].

Up to now, different groups are working to generate a C9orf72 animal model. Despite these models did not recapitulate the pathology, C9orf72 animal model are useful to understand the molecular mechanisms of C9orf72 expansion.

For these reasons, the mutant SOD1 rodent models remain the best models currently available to study the pathogenesis of ALS and to test new therapies, although the question remains how to model sporadic forms of ALS [73].

Moreover, some drugs established to be efficient in mice models have failed in clinical trials [74]. The causes for the failed translation of drug trials from animal models to humans can be due to several causes, including differences in drug penetration and metabolism that lead to a different biological response [75].

1.1.4 Molecular mechanisms

The basis of ALS are not entirely understood, and the molecular and cellular events involved in disease progression are various and complex. A wide spectrum of toxic molecular mechanisms has been proposed to mediate the progressive MN degeneration in ALS. Besides, to complicate matters further, different cell types, such as astrocytes, neurons, microglia and oligodendrocytes, contribute to the development of this pathology [76] (Fig. 3).

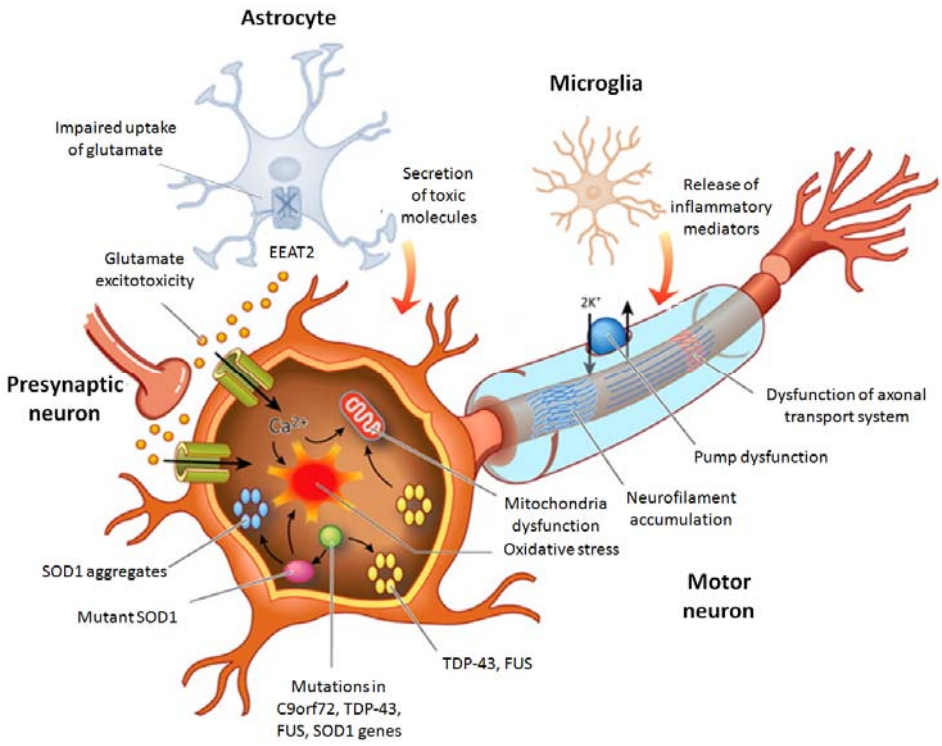

Fig. 3 Representation of pathophysiological mechanisms underlying ALS.

The mechanisms that underlie the development of neurodegeneration in ALS are multifactorial, with evidence of a complex interplay between genetic mutations and dysfunction of molecular processes.

The proposed molecular mechanisms of ALS include:

Aggregation of toxic proteins

Numerous WT and mutated proteins are dysfunctional in both sporadic and familial forms of ALS. Anomalies in physiological homeostasis of proteins are shown by presence of toxic aggregates, impaired degradation, abnormal cleavage events and distinctive post-translational modifications like ubiquitination and hyperphosphorylation [77].

Aggregates of mutant proteins are hallmarks of neurodegenerative diseases, including ALS. Intracellular aggregates seem to alter the homeostasis of proteins and to determine cellular stress. It is hypothesized that these deposits sequester RNA and proteins essential for normal cellular function, impairing protein degradation and interfering with axonal transport. Moreover, the energetic exhaustion can be linked to the turnover of misfolded proteins [78]. Toxic deposits of protein are found in ALS patients and animal models and consist in aggregation of SOD1, TDP-43, FUS and DPR proteins. In particular, in the spinal cord of SOD1G93A mice model, SOD1 protein undergone to an abnormal/altered conformation, conventionally named as misfolded, which may explain its innate toxic nature [33, 79-81]. These aggregates occur both as primary consequences of protein mutations and as secondary phenomena due to the underlying disease process. However, it remains unclear if they are directly toxic or whether they reflect a cellular response. It has also been considered the possibility that some aggregates are beneficial and compensatory events [77].

Non-cell-autonomous toxicity

In ALS, the selective degeneration of MNs led researchers to focus on the mechanism within MNs. These mechanisms are known as cell autonomous mechanisms. However, recent discoveries reveal that also non-cell autonomous processes can significantly contribute to MN dysfunction and death [82]. In particular, astrocytes and microglial cells that surround MNs appear to play fundamental roles in ALS onset and progression [83]. Degenerating MNs release neurotoxic molecules that stimulate surrounding cells to produce reactive oxygen species and pro-inflammatory cytokines, resulting in MN damage and initiating the vicious cycle of progressive cell death [84]. Among the activated cells, astrocytes and microglia shift their function from anti-inflammatory and neuroprotective to pro-inflammatory and neurotoxic.

Microglial cells, which are derived from the hematopoietic cell lineage, are the primary innate immune cells of the central nervous system. In ALS, microglial cells release reactive oxygen species, proinflammatory cytokines and other toxic substances, accelerating the MN death [85].

Astrocytes, which are of neuroectodermal origin, do not belong to the immune system but may take part in the immune response, particularly in pathological states. In healthy condition, astrocytes produce neurotrophic factors that preserve and support neurons and maintain low concentration of glutamate removing the excess from the extracellular space [82]. In ALS, astrocytes lose their physiologically functions inducing neuronal death by loss of neurotrophins and reduced up-take of glutamate. Moreover, *in vitro* studies show that astrocytes release neurotoxic factors that can induce apoptosis in MNs. One of the identified toxic factors is neurotrophic growth factor [86].

Alteration of axonal transport

An efficient axonal transport is critical event for neuronal function. There are well-demonstrated defects of axonal transport in both ALS animal models and human patients [87]. Researchers have identified mutations related to ALS in genes encoding for proteins directly involved in the axonal transport system [88, 89]. However, in the majority of ALS patients, altered axoplasmic flow is probably a secondary effect. Impaired axonal transport can be caused by alteration in key pathways, including the glycogen synthase kinase 3 beta (GSK3β) pathway. It has been demonstrated that GSK3β is a kinase that regulates neural polarization, neuritogenesis and axonal growth [90]. In ALS, GSK3β is overexpressed and hyperactivated inducing MN death.

Oxidative stress and mitochondrial dysfunction

There are substantial evidences implicating oxidative stress and mitochondrial dysfunctions as pathogenic mechanisms of ALS [91]. While the first hypothesis proposed for SOD1 mutations suggested a primary role in oxidative stress related to its superoxide dismutase function, it is now presumed that the oxidative stress is only a secondary component of the pathogenesis.

Post mortem tissues of ALS patients reveal clear signs of oxidative stress, as shown by a widespread accumulation of oxidative damage to proteins, DNA and lipids. Oxidative stress may cause several toxic effects such as formation of toxic misfolded protein, damage of mitochondria and activation of abnormal pathways [92]. Damages to mitochondria caused by oxidative stress induce alterations in morphology and function, resulting in energy deficit, calcium accumulation and activation of death pathways.

Among apoptotic pathways, the GSK3β pathway seems to be involved in neuronal degeneration. In fact, once activated, GSK3β induces activation of a series of substrates that causes apoptosis via cytocrome c caspase [93].

Alterations of RNA metabolism

A surprising number of proteins associated to ALS are directly or indirectly involved in RNA processing [94]. For this reason, currently the main theory to explain the aetiopathogenesis of ALS is referred to alterations in RNA metabolism [87]. This is due to the finding of mutations in genes encoding for DNA and RNA binding proteins, like TDP-43 and FUS, which are directly involved in every aspect of RNA metabolism, such as transcription, RNA splicing and transport, miRNA processing and translation [95]. Furthermore, one of the three molecular mechanisms postulated for C9orf72-associated ALS consists in toxic gain of function of abnormal messenger RNA. Expanded C9orf72 mRNA forms RNA foci that sequester critical RNA-binding proteins, which are then unable to correctly splicing other mRNAs [87]. Recently, a convergence of molecular pathways involved in RNA metabolism is emerging for ALS [96].

Although several advances in investigations has led to the identification of cellular and molecular processes involved in the MN degeneration, the understanding of the origin and progression of the ALS is still largely insufficient.

1.1.5 Therapeutic strategies

The impact of ALS is tremendous not only on health and quality of life of patients, being severely disabling and fatal, but also on their family and society. For these reasons, ALS is associated with large unmet medical needs, in particular the urgency of an efficient, clinically meaningful therapy. To date, Riluzole is the only therapeutic drug approved by the U.S. Food and Drug Administration as a safe and well-tolerated treatment for patients with ALS. Riluzole is a benzothiazole anticonvulsant that reduces excitotoxicity by blocking voltage-gated sodium channels, with a consequent decrease in the pre-synaptic release of glutamate [97]. Riluzole modestly prolongs survival by 2-3 months, delaying the use of supportive approaches, such as tracheostomy and mechanical ventilation.
Because the beneficial effects of Riluzole are very modest with a small beneficial effect on both bulbar and limb function, but not on disease progression, effective treatments are urgently needed [98].
The knowledge of molecular and cellular mechanisms underlying ALS can be useful to develop an effective cure for this fatal disorder. The therapeutic strategies under development for ALS can be divided in pharmacological approaches, gene and cellular therapies (Table 2).

Table 2. Therapeutic strategies for ALS

Compound	Type of approach	Target	Mechanism of action	Phase
Dexpramipexole	Pharmacological (small molecule)	Mitochondria	Reduction of oxygen consumption	III failured
Rasagiline	Pharmacological (small molecule)	Mitochondria	Enhancement of mitochondrial viability	II
Fingolimod	Pharmacological (small molecule)	T cells	Blocking of T cells in lymph nodes	II
NP001	Pharmacological (small molecule)	Macrophages	Reduction of macrophage activation	II
Tocilizumab	Pharmacological (antibody)	Macrophages	Reduction of macrophage activation	II
Lithium Carbonate	Pharmacological (small molecule)	Autophagic cells	Boosting of autophagy-clearing misfoled proteins	II
Masitinib	Pharmacological (small molecule)	Mast cells	Blocking of cytokine production and mast cell migration	III
Fasudil	Pharmacological (small molecule)	Astroglial and microglial cells	Reduction of astroglial and microglial cell infiltration	II
Ozanezumab	Pharmacological (antibody)	NMJs	Repairing of motor nerves and muscle fibers	II
Tirasemtiv	Pharmacological (small molecule)	Fast skeletal muscles	Modulation of muscle contractility	III
Ibudilast	Pharmacological (small molecule)	Glial cells	Suppression of glial cell activation	II
RNS60	Pharmacological (small molecule)	Neurons and glia	Reduction of inflammation	II
Tamoxifen	Pharmacological (small molecule)	Neurons	Enhancement of autophagy pathway	I/II
Memantine	Pharmacological (small molecule)	Neurons	Reduction of excitotoxicity	II
Retigabine	Pharmacological (small molecule)	Neurons	Reduction of excitability	II

Compound	Type of approach	Target	Mechanism of action	Phase
BIIB067 (Isis-SOD1Rx)	Gene therapy	Neurons and glia	Reduction of misfolded SOD1	I
Antisense C9orf72	Gene therapy	Neurons and glia	Reduction of C9orf72 inclusions	Preclinical
Glial restricted progenitor cells (Q Cells)	Cell therapy	Astrocytes	Replacement of toxic astrocytes	I/II
Bone Marrow Derived Stem Cell	Cell therapy	Neurons and glia	Cell replacement and micro-environmental enrichment	I/II
Adipose Derived Mesenchymal Stem Cells	Cell therapy	Neurons and glia	Cell replacement and micro-environmental enrichment	I
Neural Stem Cells	Cell therapy	Neurons and glia	Cell replacement and micro-environmental enrichment	II

Pharmacological approaches

Pharmacological approaches include antibodies and small molecules that act on different targets with different molecular mechanisms of action. These drugs can be therapeutic via mitochondria protection, reduction of neuroinflammation and inhibition of kinase involved in aberrant pathways. Some of them, such as Masitinib and Tirasemtiv, are now in phase III clinical trial, or have been recently tested like Dexpramipexole (www.clinicaltrials.gov).

Dexpramipexole reduces MN death by inhibiting aberrant mitochondrial leak conductance. Although Dexpramipexole seems to have positive effects in the phase II trial, the phase III study revealed an inefficacy of Dexpramipezole on survival, improving symptoms and disease progression.

The negative phase III results suggest that the phase II clinical trials for ALS might need to be redesigned (ClinicalTrials.gov Identifier: NCT01281189).

Masitinib is a c-kit tyrosine kinase inhibitor, already evaluated to treat a wide range of diseases, including multiple sclerosis, Alzheimer's disease and some types of cancer. The inhibition of c-kit, a stem cell factor receptor, causes a block of the proliferation and activation of mast and microglial cells. A phase III study is now ongoing and the interim results seems positive (ClinicalTrials.gov Identifier: NCT02588677).

Tirasemtiv is a selective troponin activator that directly modulates the contractility of muscles, increases the strength of different skeletal muscles, particularly those crucial for breathing. Tirasemtiv resulted to be safe and tolerated in phase II clinical trial and a phase III is now ongoing (ClinicalTrials.gov Identifier: NCT02936635).

Gene therapy

A different approach to treat ALS consists in the injection of short synthetic and chemically modified nucleic acid that binds to an mRNA target, interfering with its function. This approach known as molecular therapy is now ongoing in clinical trials for several disorders (www.clinicaltrials.gov). For ALS, a possible gene therapy consists in delivery of antisense oligonucleotides to reduce the expression of toxic/misfolded proteins, such as SOD1 and C9orf72 [46, 81, 99].

Pre-clinical studies performed on rodent model of ALS have been showed a reduction of SOD1 aggregates and increased survival after the treatment with an antisense oligonucleotide that targets SOD1 gene.

This antisense oligonucleotide (ISIS-SOD1RX) is now tested in phase I clinical trial in patients with SOD1-associated fALS through intrathecal administration into the spinal cord. This therapy reduces SOD1 levels and seems to be safe and tolerable [99].
To treat C9orf72 repeat expansion, preclinical studies with antisense oligonucleotides are now ongoing [46].

Cell Therapy

Cell therapies are receiving increasing interest as tool to treat numerous human neurological conditions, including traumatic injuries and neurodegenerative diseases [100]. For ALS, the cell therapies up to now investigated include the transplantation of mesenchymal stem cells, which are derived from adipose tissue or bone marrow, human neural stem cells (NSCs), and glial restricted progenitor cells.
As this thesis is focused on NSC transplantation, the stem cell-based approach for ALS treatment will be analyzed in detail in the next chapter. These strategies can be therapeutic through multiple mechanisms, which include not only cell replacement but also a reduction neuroinflammation, production of neurotrophic factors, and reduction of aggregates [101].
According to the website www.clinicaltrials.gov, there are currently 22 ongoing clinical trials using transplantation of various stem cell types. The number of trials is elucidative of the rapid progress in the field with the prospective to finally find an effective treatment for this still incurable disease.

1.2 Stem Cells

1.2.1 Induced Pluripotent Stem Cells (iPSCs)

Stem cells are undifferentiated cells characterized by the ability to self-renewal, giving rise indefinitely to more cells of the same cytotype by symmetric division, and to differentiate in mature cells through asymmetric division [102]. Thanks to their potential of differentiation, stem cells could be divided in totipotent, pluripotent and multipotent. Totipotent cells are able to differentiate in whatever kind of cells; they are in the first few cell divisions in embryonic development. After four days, the cells begin to specialize into pluripotent stem cells. Pluripotent cells are able to generate every cytotypes but they cannot give rise to an entire organism, they are in the inner cell mass of blastocystis. Multipotent cells are tissue specific and could differentiate in a limited number of cytotypes.

Among stem cells, the most used in pre-clinical studies are pluripotent stem cells derived from embryos, known as embryonic stem cells (ESCs).

The use of ESCs-differentiated cells, however, is complicated because of the problems of possible rejection if transplanted since they are heterologous, and because of ethical issues; in fact, the creation embryonic stem cells imply the destruction or manipulation of the pre-implantation stage embryos [103]. In order to overcome these obstacles, recently an important breakthrough has been made with the generation of stem cells derived from adult somatic cells. These cells are known as induced pluripotent stem cells (iPSCs) [104].

The iPSCs are generated by reprogramming differentiated adult cells to a state of pluripotency, through the expression of transcription factors. iPSCs were first generated in 2006 from mouse fibroblasts [105], afterwards the iPSCs were generated from human cells in 2007 [106].

To reprogram adult cells into iPSCs, Takahashi and Yamanaka used 4 genes encoding key transcription factors with retroviral vectors. In particular, skin fibroblasts were transfected with Sox2, Oct4, c-Myc and Klf4 [105, 106]. Currently, several studies improved the protocol for iPSC generation in terms of time and efficiency [104, 106-108]. The current methods for iPSC generation can be divided into three categories based on the vector types: virus (retrovirus, lentivirus, adenovirus, Sendai virus), DNA (plasmid, episomal plasmid, transposon) and cell-penetrating peptide. Because viral vectors have high efficacy of reprogramming, but present a major risk of tumor formation, many studies apply the non-viral method developed by Yu and colleagues in 2007 to generate iPSCs [104, 109-111]. They used a non-viral, non-integrating method for iPSCs generation through the transfection of 6 reprogramming factors vehiculated by episomal vectors. In particular, six reprogramming factors (OCT4, SOX2, NANOG, LIN28, c-Myc and Klf4) were conveyed through OriP/EBNA1 carriers. This technique decreases the modifications in the recipient cells, making them more similar to physiological stem cells, and has a satisfactory efficiency [109].

The iPSCs show genetic and morphological features similar to the ESCs. Like ESCs, the iPSCs grow as colonies in adhesion and form spheres named embryoid bodies (EBs) when resuspended in low attached plates in appropriate medium. They also express the typical marker of pluripotency such as TRA1-60, TRA1-81, SSEA3, and SSEA4.

The iPSCs maintain the potential of self-renewal and can differentiate into the three germ layers *in vitro* and *in vivo*, forming teratomas when transplanted in immunodeficient mice [112].

Since iPSCs are directly derived from adult tissues, they not only bypass the issue of the embryo manipulation, but they can be made in a patient-matched manner [113].

This means that each patient could have own iPSC line that can be used for autologous transplants without the risk of immune rejection. Moreover, the patient-specific iPSCs are an abundant and important source for the generation of specific cells directly involved in the pathology.

For these reason, the iPSC technology is a promising tool, not only because it represents a rather novel source of autologous stem cells for the transplantation, but also to model human pathology *in vitro* (Fig. 4).

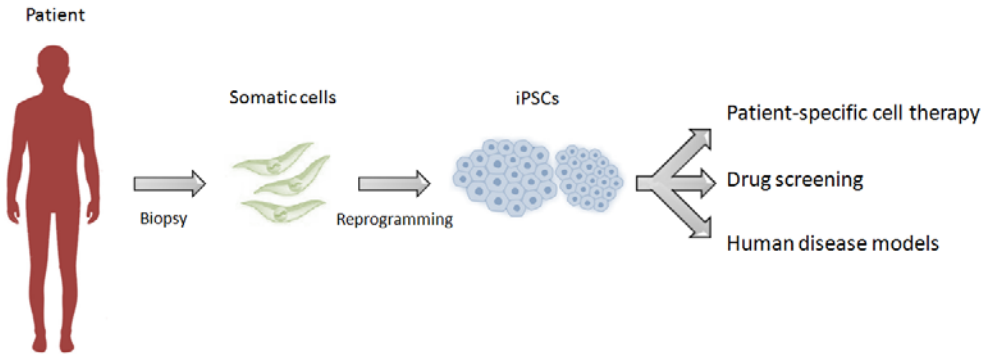

Fig. 4 Potential applications for iPSCs.

Generation of iPSCs is obtained by reprogramming adult somatic cells derived from biopsy of healthy or affected patients. Once generated, iPSCs are well suited to modeling human pathologies *in vitro* and screen drugs. Moreor, iPSCs are promising for regenerative medicine and can be used for autologous transplants.

1.2.2 iPSCs as an *in vitro* model of ALS

Since ALS is an adult-onset disease characterized by high heterogeneity and few animal models exist, the iPSCs technology for ALS is particularly promising [114]. In fact, although animal models are useful and informative on the mechanisms behind ALS pathogenesis, their use requires months of studies, high costs and currently there are no models for some genetic mutations and sporadic forms of ALS. This leads researchers to focus on iPSCs technology to better understand the precise pathways causing MN death and to evaluate the effects of different drugs and therapies.

Recently, iPSCs have been generated from patients carrying mutations associated to familial cases of ALS as well as from sporadic patients [115]. This technology represents a reliable model for ALS since the disease-affected cells harbor the patients' genomic backgrounds.

Because patient-specific, the iPSCs can recapitulate *in vitro* the pathology, allowing us to understand the mechanisms of ALS and the differences/analogies among sporadic and familial forms of ALS and between different mutations. The iPSCs are particularly useful to model the ALS-related mutations that do not have a correspondent animal model such as C9orf72 and sporadic cases of ALS. The iPSCs can be derived from both fALS and sALS and are versatile in allowing investigators to differentiate these cells into multiple cell types, such as different types of specialized neurons as well as supporting glial cells [114]. This aspect is very important for ALS, where affected cells can be derived only post-mortem from the CNS representing a scarce source of cells that reflect the end-stage momentum of the disease.

The iPSCs can efficiently model *in vitro* ALS thanks to their ability to differentiate and generate the affected cytotypes.

They can differentiate using multi-step protocols in both upper and lower MNs and in surrounding astrocytes.

To generate lower MNs from iPSCs, several protocols exist and all of them are based on knowledge of what happen *in vivo*. During embryogenesis, the neuroectodermal fate is determined by dual inhibition of bone morphogenic proteins (BMPs) and transforming growth factor beta (TGFβ) pathways that induce respectively mesoderm and endoderm fate.

In vitro, neuralization of iPSCs is specified by inhibition of BMPs and TGFβ signaling by small molecules SB31542 and LDN193189. These molecules are added early during iPSC differentiation and they selectively block endodermal and mesodermal fate [116], determining the formation of NSCs. NSCs are multi-potent cells characterized by the capability to self-renewal and to differentiate into neurons, astrocytes and oligodendrocytes [117]. To generate spinal MNs, which are positioned in the ventral horn of spinal cord, it is required the caudalization and ventralization of NSCs. For this reason, NSCs are treated with Retinoic acid (RA), which induces caudal neuronal subtypes of the hindbrain and rostral spinal cord and further directs neurogenesis, and Sonic Hedgehog (Shh), which determines dorsoventral spinal identity [118].

The differentiation of iPSCs derived from ALS patient into lower MNs of the spinal cord was first reached by Dimos and colleagues in 2008 [119]. They used skin fibroblasts derived from ALS patient with an autosomal dominant SOD1L144F mutation, which were reprogrammed to form iPSCs and then differentiate into lower MNs using a combination of Shh and RA. They showed a similar ability of these iPSCs to form MNs compared to ESCs. The efficiency of this protocol wasn't high; in fact, they obtained less than 20% of MNs, as revealed by their expression for the marker Hb9.

Currently, several studies improved the protocol for MN generation in term of time and efficiency, based on combination of small molecules that accelerate their formation within 3 weeks [120].

Although iPSC have the potential to differentiate in upper MNs, their generation *in vitro* is complex and more difficult compared to lower MN production. The existing protocols are not yet capable of generating specific classes of cortical neurons, including upper MNs [118]. One of attempts for upper MN generation consists in the conversion of iPSC into cortical progenitor cells in the absence of added morphogens and prolonged presence of the BMP inhibitor [121].

The iPSCs can also generate astrocytes in 90 days, resulting in cells with typical features of physiological astrocytes such as positive expression for GFAP, EEAT2 (excitatory amino acid transporter 2) and acquaporin 4 and able to secrete neurotrophic factors (BDNF, GDNF). Moreover, if co-cultured with MNs, the iPSC-derived astrocytes enhance their survival and axon growth [122].

iPSC-derived cultures can mirror pathology observed in post-mortem patient neural tissues, indicating that this technique accurately represents cellular phenotype and that iPSCs are a useful tool for uncovering disease implications.

Increasingly experiments are now performed using iPSC-derived MNs generated from ALS patients carrying mutations in SOD1, TDP-43, C9orf72, as well as sporadic ALS subjects (Table 3).

Table 3. iPSC-derived MNs from sALS and fALS patients.

Mutation	Phenotypes and mechanisms observed in iPSC-derived neurons	Drug screening	References
$SOD1^{D90A}$	Neurofilaments aggregates and inclusions; Mutant SOD1 binds and destabilizes neurofilament mRNA	TALEN-mediated homologous recombination	[123]
$SOD1^{A4V}$ (C9orf72?)	Increased apoptosis; Reduced soma size; Shorter and fewer processes; Defects in mitochondrial morphology; Unfolded protein response and ER stress	Zinc finger nuclease (ZFN) correction of $SOD1^{A4V}$ mutation improves survival and soma size	[124]
$SOD1^{G93A}$ $SOD1^{L144F}$	Improved motor neuron survival by inhibition of GSK3α/β and Tak1-MKK4-JNK-c-Jun	Small molecules screening	[125]
$SOD1^{A4V}$ $SOD1^{D90A}$ $SOD1^{G85S}$ C9orf72 FUS^{M511FS} FUS^{H517Q}	Hyperexcitability blocked by Retigabine	-	[126]
$TDP\text{-}43^{M337V}$	Cellular vulnerability to PI3K inhibitors; Increased detergent-insoluble TDP-43 proteins	Specific siRNA reduces cytosolic TDP-43	[127-129]

Mutation	Phenotypes and mechanisms observed in iPSC-derived neurons	Drug screening	References
TDP-43^{Q343R} TDP-43^{M337V} TDP-43^{G298S}	Reduced neurofilament expression; Increased detergent-insoluble TDP-43; Increased TNFα/NFκB signaling pathway and sensitivity to arsenite-induced cell death; Increased RNA metabolism-related genes	Anacardic acid decreased TDP-43 mRNA, reduced insoluble TDP-43 and increased neurofilament expression	[130]
TDP-43^{A90V} TDP-43^{M337V}	Cytoplasmic localization of TDP-43 protein; Reduced total TDP-43 protein; Decreased miR-9 and precursor	-	[131]
TDP-43^{M337V} TDP-43^{G298S} TDP-43^{A315T}	Axonal transport of target mRNAs	-	[132]
C9orf72	RNA foci and RAN translation products; Screening for RNA binding proteins that bind GGGGCC and characterize ADARB2; Gene expression profiling; Increased glutamate cytotoxicity	ASOs targeting C9orf72 rescued glutamate cytotoxicity and reversed disease-specific transcriptional changes	[44]
C9orf72	RNA foci detected but not RAN products; Support gain-of-function properties in RNA foci to sequester RNA binding proteins and affect splicing and transcription; Gene expression profiling revealed enrichment in genes related to cell adhesion, synaptic transmission and reduced excitability upon depolarization	ASOs targeting C9orf72 reversed disease-specific transcriptional changes	[47]

Mutation	Phenotypes and mechanisms observed in iPSC-derived neurons	Drug screening	References
C9orf72	RNA foci and RAN translation products; Reduced cell viability in the presence of autophagy inhibitors	-	[45]
C9orf72 TDP-43^{M337V}	Initial hyperexcitability, followed by a progressive loss in action potential and synaptic activity	-	[133]
C9orf72	Decreased cell survival correlated with dysfunction in Ca^{2+} homeostasis; Reduced levels of the antiapoptotic Bcl-2; Increased endoplasmic reticulum stress; Reduced mitochondrial membrane potential; Abnormal protein aggregation; Stress granule formation	-	[134]
C9orf72	RanGAP physically interacts with RNA and is mislocalized; Nuclear import is impaired	Small molecules and ASOs targeting the HRE G-quadruplexes	[135]
FUS^{H517D}	Mis-localization of FUS; Stress granules Cellular vulnerability; Aberrant gene expression and/or splicing pattern	-	[136]
Sporadic ALS	Genetic analysis indicated association between mitochondrial function and cellular processes	-	[137]
Sporadic ALS	TDP-43 protein aggregates in iPSC-derived motor neurons from sporadic ALS cases	High content screening for TDP-43 aggregation inhibitors	[138]

In particular, iPSC-MNs with SOD1 mutations showed a high propensity to die via apoptosis, reduction in term of soma size and in number of neuronal processes, which are also shorter compared to healthy MNs. Moreover, genome wide analyses revealed altered expression in genes implicated in cytoskeleton organization, mitochondrial function and structure and unfolded protein response. More recently, researchers found in iPSC-MNs possessing SOD1 mutations the presence of neurofilament aggregation and neurite degeneration [123].

iPSC-derived MNs carrying TARDBP mutations have showed increased levels of soluble and detergent-resistant TDP-43 and reduced survival. Gene expression profiling in iPSC-derived MNs revealed misregulation of genes linked to RNA metabolism and cytoskeleton functions [130].

Similar to those reported in SOD1 MNs, iPSC-derived MNs with TDP-43 mutation showed a significantly reduction in neurofilament expression. Motor neurons derived from differentiation of iPSCs with C9orf72 repeat expansion well recapitulated some of the neuropathological hallmarks observed in ALS patients. Several groups showed the co-localization of repeat mRNA with RNA binding proteins, which form intranuclear RNA foci, altered gene expression patterns and RAN translation products in iPSC-derived MNs [44, 47, 139]. Also, C9orf72 iPSC-derived neurons provided a convenient tool to investigate the potential loss-of-function effect of C9orf72 gene product in the regulation of endosomal trafficking and in the pathogenesis of TDP-43 proteinopathy [140, 141]. Moreover, iPSC-neuronal cultures from C9orf72 patients have contributed to identification of nuclear RNA export defects [142] and nuclear import impairment caused by nucleocytoplasmic transport protein mislocalization [135].

An important benefit of iPSC cultures consists in the opportunity to model disease when the genetic causes are unknown, such as in sporadic ALS forms. In 2013, Burkhardt and colleagues identified intranuclear TDP-43 aggregates in different lines of iPSC-derived MNs from sALS patients without mutations in genes already known to be linked in ALS TDP-43 proteinopathy [138].

Finally, iPSCs provide a platform for the investigation of therapeutics; the screening of new compounds in iPSC derived from ALS patients can be useful to find an effective cure. For example, to correct SOD1A4V/+ mutation, researchers used gene targeting approach mediated by zinc finger nuclease (ZFN) which showed significant rescues in both survival and soma size in corrected MNs [124]. Similar approach (mediated by TALEN-based homologous recombination) was used to correct the SOD1D90A mutation [143].

Drug screening assays performed on TDP-43 iPSC-derived MNs led researchers to identify a histone acetyltransferase inhibitor (anacardic acid) as a potential treatment for ALS. In fact, treatment with anacardic acid determined a reduction of the insoluble TDP-43 resulting in an enhancement in neurite length and in expression of neurofilaments. Anacardic acid also down-regulated the RNA metabolism-related genes and reversed the changes in apoptotic pathway [130].

Two studies revealed that the treatment with antisense oligonucleotide (ASOs), that targets the GGGGCC repeat, mitigated C9orf72 disease characteristics in iPSC derived cultures [44].

Challenges

Challenges with iPSC technology are various. First of all, the efficiency of iPSC reprogramming is often low, less than 0.02% in fibroblasts [106] costing time and resources.

Another important aspect to be considered for basic and translational studies is the possibility to create iPSCs free from exogenous sequences and from genes involved in the reprogramming process. This is now possible thanks to the use of episomal vectors and the Sendai virus, which remain episomal and not integrating into the host genome [144]. However, the transfection efficiency is reduced due to antiviral response of cells. During or after reprogramming, chromosomal abnormalities can occur, causing potential effects on the cellular phenotypes [145]. For this reason, karyotype analysis of iPSCs is considered a standard for ensuring that findings are linked to ALS and not as a result of chromosomal alterations. Moreover, the comparison of results from lab to lab can be challenging due to the multiplicity of differentiation protocols. The standards to define iPSC-derived MNs or astrocytes consist on the identification of a small subset of specific markers, such as Hb9 and GFAP respectively. In addition, the purity of cultures derived from iPSC is always of concern. In fact, population studied can be heterogeneous determining non-cell autonomous effects that influence the experimental results [114].

Finally, since cultures generated from iPSC do not reach fully mature phenotypes, a relevant concern is whether iPSC derived MNs adequately model late onset diseases, like ALS, making it challenging to study adult late-onset neurodegeneration. To bypass this issue, an age mimicking gene (progerin) was introduced in cells in order to accelerate the aging process [99]. Despite the necessity to improve the reprogramming process, this technology remains an important tool to model ALS, contributing to an increasingly robust research system [146].

1.2.3 Stem cell transplantation as therapy for ALS

Given the multifaceted nature of ALS, the therapy based on transplantation of differentiated cells from ESCs and iPSCs has recently become an attractive option. Since many pathological mechanisms appear to contribute in ALS onset, the therapeutic strategies that targeted only one mechanism may have minimal impact on disease phenotype [147]. On the contrary, approaches that affect many pathophysiological mechanisms at the same time could lead to a therapeutic synergy.

Among strategies that can be therapeutic through multiple mechanisms there is the transplantation of stem cells. In fact, although the initially proposed mechanism was cell replacement, now it is clear that stem cells may also provide a large number of benefits by modulating the micro-environment, leading to a reduction of inflammation and protecting MNs and neuronal circuitry [148].

Thanks to the ability of stem cells to differentiate in neuronal subtypes lost in ALS, transplanted stem cells-derived neurons can restore MN function through cell replacement.

After transplantation into animal models with MN injury, stem cell-derived MNs are able to extend axons and form NMJs with host muscles, giving rise to a partial recovery from paralysis [149-152].

Despite the success of stem cell-based approaches in ALS animal models [153], some issues of direct MN replacement limit the clinical translation in human. In fact, in order to functionally replace degenerating MNs, the engrafted stem cell-derived neurons must receive functional synapses and send axons over long distances to the target muscles and form NMJs. Due to these limitations, the direct replacement of MN populations seems unlikely to be a viable treatment option in the short term for ALS.

Therefore, it is evident that the mainly beneficial effect derived from transplantation of stem cells consists in modulation of the micro-environment rather than cell replacement [154].

Stem cells transplantation determines environmental enrichment, counteracting MN loss, by releasing neurotrophic factors, eliminating toxic factors, reducing inflammation and generating auxiliary neural networks around affected areas. These effects determine a more realistic near-term clinical goal for ALS.

Among the trophic factors produced by stem cells, glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I), and vascular endothelial growth factor (VEGF) protect and support neurons from degeneration [155].

To enrich and modulate neural micro-environment, many strategies use stem cells to furnish de novo synthesized neurotrophins and to delivery them to the foci of disease [156]. It is also possible to manipulate stem cells in order to make them able to produce trophic factors.

Over the last decade, many preclinical studies have been conducted with the aim to test the therapeutic effects of stem cells transplantation, using various types of stem cells and different routes of administration (Table 4).

Table 4. Stem cell transplantation in preclinical studies

Type of Stem Cells	Animal model	Additional treatment	Results	Reference
Human embryonic germ cells	Rats with motor neuron injury induced by sindbis virus	-	Blood brain barrier improvement, Limb strength	[157]
Mouse embryonic stem cells	Rats with motor neuron injury induced by sindbis virus	RA and Shh agonist	-	[150]
Human neural precursor cells	Mutant SOD rats	GDNF, Gene transfer	Blood brain barrier improvement	[158]
Human neural precursor cells	Mutant SOD rats	-	Blood brain barrier improvement, Extended survival	[159]
Mouse neural stem cells LeX+CXCR4+	Mutant SOD mice	-	Rotarod, Extended survival	[160]
Human immortalized neural stem cells	Mutant SOD mice	VEGF, Gene transfer	Rotarod, Extended survival	[161]
Rat neural stem cells	Mutant SOD rats	GFP labeled	-	[162]
Mouse progenitor stem cell c-kit+	Mutant SOD mice	GFP labeled	Rotarod, Extended survival	[163]
Human neural stem cells	Mutant SOD mice	Olig2 gene transfer, Shh treatment	Rotarod, Extended survival, Limb placement	[164]

NSCs in pre-clinical studies

Before stem cell therapy can move to the clinic as a treatment for ALS, some aspects have to be considered. In fact, the proper type of stem cells and their delivery must be carefully considered [148].

Compared to other types of stem cell, NSCs are particularly promising for ALS treatment. This is due to the capability of NSCs to primarily differentiate into neuronal and glial cells, determining a life-long source of nervous cells. Moreover, since more differentiated than pluripotent stem cells, NSCs reduce the risk of forming teratoma after transplantation. Different preclinical studies have demonstrated the positive effects of their transplantation *in vitro* and in ALS animal models [110, 165].

Teng and colleagues have demonstrated that NSCs derived from embryonic stem cells (mouse and human) transplanted into the spinal cords of SOD1G93A adult mice effectively improved neuromuscular performance and respiratory function, slowing ALS progression and prolonging survival. Moreover, in 25% of affected mice treated with NSCs, the ALS phenotype seemed completely rescued [165].

Given the ability to overcome ethical issues of the embryo manipulation, human iPSCs provide an alternative source of NSCs. Recently, our laboratory has demonstrated the beneficial effects of a specific subpopulation of iPSC-derived NSCs selected for their aldehyde dehydrogenase (ALDH) activity and VLA4+ positivity. This subpopulation of NSCs has been selected in order to enhance their pluripotency and migration capability. Since ALDH is a marker of pluri/multipotency, NSCs selected for this marker increase their growth/differentiative potential and ability to replace cells. The presence of VLA4 integrin in NSCs allows them to cross the blood-brain barrier (BBB), particularly in the presence of inflammation as in ALS.

When intrathecally transplanted into the SOD1G93A mice, these cells migrate into the spinal cord, improving motor performance and increasing life span [110].

Overall, preclinical studies provide promising data and could pave the way for clinical trials in humans. However, more work is necessary in order to acquire additional information about the details of stem cell-based therapy effects.

Ongoing clinical trials of NSC transplantation

On the basis of the positive results of NSC transplantation obtained in preclinical studies, the US FDA approved a "Phase I, Open-label, First in Human, Feasibility and Safety Study of Human Spinal Cord Derived Neural Stem Cell Transplantation for the Treatment of Amyotrophic Lateral Sclerosis" (NCT01348451). This trial, which is coordinated by Dr. Glass at Emory University, utilized an approach of "risk escalation" in terms of ALS severity, site and number of injection and number of transplanted NSCs. The trial design consists in 5 groups which represent both different inclusion criteria and location of surgery (Table 5).

Table 5. Design of Phase I trial of human NSC transplantation for ALS

Group	N° patients	N° of injections	Target	N° of NSCs
A1	3	5 total, 5 unilateral	Lumbar cord	5×10^5
A2	3	10 total, 5 per side	Lumbar cord	1×10^6
B	3	5 total, 5 unilateral	Lumbar cord	5×10^5
D	3	5 total, 5 unilateral	Cervical cord	5×10^5
C/E	3	15 total, 5 per side lumbar 5 unilateral cervical after observation period	Lumbar cord Cervical cord after observation period	1×10^6 5×10^5

To ensure survival of the transplanted NSCs, patients were subjected to a multi-drug regimen that includes intravenous injections of basiliximab and methylprednisolone during the first week, as well as mycophenolate mofetil and tacrolimus for 6 months.

The treatment was apparently safe. In fact, no tumor formation was evident in any subject; moreover, NSCs engrafted the host tissue, as confirmed by the presence of living cells in the injection regions. Adverse events were mostly related to the immunosuppression regimen.

So far, 7 patients enrolled in phase I trial are dead and the autopsy analyses on spinal cord tissues of 7 patients that died revealed the presence of engrafted donor cells.

Despite the purpose of phase I is not to evaluate the efficacy, the disease progression appeared to be reduced after surgical transplantation, in particular in group C/E.

Clinical evaluations such as ALS Functional Rating Scale-Revised (ALSFRS-R), quality of life and forced vital capacity exhibited no acceleration of disease; moreover, in most of the cases, hand-held dynamometry (HHD), grip strength testing (GST) and electrical impedance myography outcomes were improved.

Given these results, the FDA approved the "Phase II, Open-label, Dose Escalation and Safety Study of Human Spinal Cord Derived Neural Stem Cell Transplantation for the Treatment of Amyotrophic Lateral Sclerosis" (NCT01730716, clinicaltrials.gov). This phase aims to assess the maximum tolerated dose of NSCs, treating groups with an increasing dose of NSCs via intraspinal cord injections and measuring the same clinical aspects as the phase I trial (Table 6).

Since results of phase I/IIa trials are promising [166], the phase IIb/III trial is planned.

Table 6. Design of Phase II trial of human NSC transplantation for ALS

Group	N° patients	N° of injections	Target	N° of NSCs
A	3	10 total, 5 per side	Cervical cord	2×10^6
B	3	20 total, 10 per side	Cervical cord	4×10^6
C	3	20 total, 10 per side	Cervical cord	6×10^6
D	3	20 total, 10 per side	Cervical cord	8×10^6
E	3	20 total, 10 per side for each target	Cervical cord, followed by lumbar cord	16×10^6

The first Italian clinical trial using NSCs for ALS started in 2010. Named "Intramedullary transplantation of human neural stem cells as a putative therapy for ALS: Proposal for a Phase I clinical trial", this study was coordinated by Dr. Vescovi at the Azienda Ospedaliera Santa Maria (Terni). Six non-ambulatory patients were treated with injections into the lumbar spinal cord; three of them received unilateral injections, while the remaining ones received bilateral injections. From the point of view of clinical follow-up patients did not manifested complications related to the experimental procedure [167].

Given the promising results obtained in both preclinical and phase I/IIa trials, NSC-based approach represents a potential promising tool compared with other pharmacological and molecular treatments in MN disease field.

2. AIMS OF THE STUDY

ALS is a neuromuscular disorder characterized by the progressive degeneration of upper and lower MNs that leads to a muscular paralysis and death within 3-5 years from the onset due to respiratory failure.
Up to now, there is no effective cure for ALS beyond palliative care and Riluzole, which delays disease progression (2-3 months) without a significant impact on the prognosis.
Stem cell transplantation is a promising therapeutic tool thanks to its multiple effects; in fact, stem cells can protect endogenous MNs through the production of neurotrophic factors, the reduction of neuroinflammation and eventually the replacement of degenerating cells.
The aim of this study consists in the selection of a subpopulation of NSCs able to engraft and migrate through the nervous system parenchyma, to replace and protect degenerating MNs and to improve ALS phenotype.

In detail, the specific aims of this study are:

- Differentiation of iPSCs, derived from the reprogramming of skin fibroblasts of control subjects, into NSCs. This cell source is attractive due to its availability, pluripotency similar to ESCs, and no ethical controversy.

- Selection of a specific subpopulation of NSCs able to engraft and migrate through the nervous system. We selected NSCs for three markers: Lewis X (LeX), a glycoprotein involved in adhesion and migration of stem cells in the pre-implantation embryo; CXCR4, a chemokine receptor that increases the sensitivity of the cells to be recruited by the host spinal cord; and β1 integrin, a subunit of VLA4 receptor that allows cells to cross the blood-brain barrier.

- Injection of LeX+CXCR4+β1 NSCs in an ALS mouse model (the SOD1G93A mouse) and evaluation of their ability to engraft the central nervous system.

- Testing and monitoring the motor functions and survival of treated mice in order to determinate the effects of cell therapy.

- Analyzing any eventual changes in ALS features such as MN and axon number, NMJ preservation and neuroinflammation.

- Evaluation of the NSC effects in a human *in vitro* model of ALS (iPSC-patient specific).

- Investigation of potential molecular mechanisms linked to the NSC therapeutic effect. More specifically, we will observe whether the production of neurotrophic factors, the inhibition of pathological astrocytes activities and the modulation of GSK3β pathway can be related to the NSC protective effect on ALS phenotype.

Overall, this study aims to contribute to set the stage for clinical trials based on NSC transplantation for the effective treatment of ALS and can provide novel insight into the mechanisms underlying this approach.

3. MATERIAL AND METHODS

3.1 Generation of iPSCs and differentiation in NSCs

To generate iPSCs, we reprogrammed human fibroblasts obtained from skin biopsy of a control subject. The reprogramming was carried out by nucleofection of three oriP/EBNA1-based episomal vectors encoding for the human genes OCT4, SOX2, c-MYC, NANOG, KLF4, and LIN28 [104, 109-111]. After electroporation, fibroblasts (1.0×10^6 cells per nucleofection) were seeded onto 3×10^{-cm} dishes covered with Matrigel (BD Biosciences) in fibroblast culture medium, which was changed every other day. At day 4, we replaced fibroblast culture medium with human stem cells medium (E8, Life Technology). After 3 weeks, it was possible to identify first iPSC colonies with stem cells morphology. We picked colonies that were morphologically similar to ES cells to further analyze and expand them. Then iPSCs were differentiated into NSCs using a multistep protocol of 10 days [110]. On day 0 of neural differentiation, the iPSC colonies were detached and put in suspension in order to form EBs in human stem cells medium for four days. Then the medium was switched to the neural differentiation medium (composed by DMEM/F12, N2 supplement, Neaa, and 1 mg/ml heparin). On day 7, the iPSC aggregates were collected and transferred into a 60^{-mm} Petri dish in neural differentiation medium. At day 10, we obtained a neuroectodermal lineage of NSCs.

3.2 Isolation of the LeX+CXCR4+β1 NSC subpopulation

After differentiation of iPSCs into NSCs, we selected NSCs for three markers. First of all, we enriched the population of CXCR4 positive cells using a magnetic cell separation strategy based on the CXCR4 MicroBead Kit (Miltenyi Biotech), following the manufacturer's instructions. Further, we evaluated the percentage of CXCR4+ NSCs with FACS. In order to distinguish dead cells, 10 millions cells/tube were incubated with 5 µl of AAD (Becton Dickinson Biosciences, BD) and with CXCR4-APC (mouse, 1:10, Miltenyi) for 10 minutes at 4°C, according to manufacturer's instructions. The CXCR4+ NSC subpopulation was re-seeded and expanded in the same neural medium. Then, the cells were sorted for their expression of integrin β1 (CD29-PE, mouse 1:50, Miltenyi) by FACS. The subset of NSCs CXCR4+β1+ were plated and expanded. The final population was further selected by FACS for their positivity for Lewis X, CXCR4 and β1 (antibodies and condition above described plus the LeX-FITC mouse, 1:10, Miltenyi). As negative control, we used the same isotypic Ig antibody. Flow cytometric sorting was performed using FACS Vantage Aria (BD Biosciences) [110]. For cell expansion and characterization, LeX+CXCR4+β1+ NSCs were growth in neuronal medium containing FGF-2 [110].

3.3 Immunocytochemistry of iPSCs and their derivates

The staining protocol for iPSCs and their derivates consisted in fixation with 4% paraformaldehyde (PFA) for 10 minutes, permeabilization with 0.25% Triton X-100 and blocking for 1 hour at room temperature with 10% BSA and 0.3% Triton X-100 in 1× PBS.

We incubated the cells with primary antibodies overnight at 4°C and with secondary antibodies for 1.5 h at room temperature (Alexa Fluor 488 or 594-conjugated anti-mouse, rabbit or goat, 1:400, Life Technologies). We used the following primary antibodies: SSEA-3 (mouse, 1:100, Chemicon), SSEA-4 (mouse, 1:500, Chemicon), TRA1-60 (mouse, 1:500, Chemicon), TRA1-81 (mouse, 1:500, Chemicon), LeX (CD15-FITC BD, according to manufacturer's instructions), CXCR4-APC and PE (Miltenyi, according to manufacturer's instructions), CD29-PE (mouse, 1:50,Miltenyi), Pax6 (mouse, 1:100, Thermo fisher), Nestin (rabbit, 1:200, Millipore), SOX2 (mouse 1:1000, Millipore), TuJ1 (rabbit, 1:300, Millipore), GFAP (rabbit, 1:300, Sigma), SMI-32 (mouse, 1:100, Millipore), Hb9 (rabbit, 1:200, Millipore), ChAT (goat, 1:125, Millipore), MAP2 (mouse, 1:100, Sigma), O4 (mouse, 1:200, Millipore). Acquisition of images was realized using the confocal LEICA LCS2 microscope.

3.4 Animal models

For our *in vivo* experiments, we employed transgenic mice of the strain B6.Cg-Tg(SOD1-G93A)1Gur/J which carries a high copy number of the mutant human SOD1 gene containing the Gly93Ala (G93A) mutation in the genome. Transgenic mice were imported by Jackson Laboratory and all experiments performed with animals were approved by the University of Milan and Italian Ministry of Health, and are in compliance with US National Institutes of Health Guidelines.

3.5 Genotyping

Progeny was obtained by breeding SOD1G93A males with C57BL/6 WT female mice. All newborn mice were genotyped using polymerase chain reaction (PCR). Genomic DNA was obtained from excision of a few millimeters from the end of a mouse's tail. To extract DNA, tails were soaked at 55°C overnight in TNES buffer and proteinase K. To precipitate DNA, NaCl and ethanol were added. The DNA was resuspended in TE buffer and amplified by PCR. SOD1 gene was amplified by the Expand Long Template PCR System (Roche Diagnostics GmbH, Mannhein, Germany) with the forward primer 5'- CAT CAG CCC TAA TCC ATC TGA-3' and the reverse primer 5'-CGC GAC TAA CAA TCA AAG TGA-3'. An internal positive control was amplified with forward primer 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3' and reverse primer 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'.

3.6 Transplantation of LeX+CXCR4+ β1 NSCs

Before transplantation, donor NSCs were genetically engineered in order to express the GFP reporter [109]. Cells (1 x 10^6) or vehicle only (saline solution) were administered into 90 days old SOD1G93A mice by intrathecal injection (5 µl). Infusions were performed between L1-L2 regions of the spinal cord using an apposite syringe (Hamilton, 30 gauge). Treatment has no collateral effects. Experimental groups consist in 12 mice (6 males) treated with NSCs and 15 mice (8 males) vehicle-treated. After treatment, all groups of mice were daily monitored up to the end stage for clinical signs of ALS, survival and histological evaluation of donor cell phenotype by blind examiners. For the entire length of the experiments, all animals received the immune suppressor FK506 intraperitoneally (1.0 mg/kg).

Littermates were equally distributed among the NSC- and vehicle-treated group and the gender was balanced. To perform histological analyses and quantification of MNs and axons, we used another group of animals at end stage (n= 6 mice per condition) [110].

3.7 Tissue analysis

At the end stage of the disease, animals were euthanized and tissues were collected for histopathological analysis. Tissues were fixed for 24 hours in 4% PFA, and soaked in 10% sucrose solution in PBS 1X (pH 7.4) overnight. Then tissues were frozen in isopentane cooled with liquid nitrogen and cryosectioned. Every tenth sections (20 µm) was collected, mounted on gelatinized glass slides and analyzed.

For immunostaining, all sections were blocked for 1 hour at room temperature with 10% BSA in 1× PBS and 0.3% Triton X-100.

In order to analyze the acquired phenotype of GFP+ transplanted NSCs, spinal cord sections were stained overnight at 4°C with primary antibodies: NeuN (mouse, 1:200, Chemicon), TuJ1 (mouse, 1:200, Chemicon), ChAT (rabbit, 1:100, Chemicon), SMI-32 (mouse, 1:100, Millipore), Nestin (mouse, 1:200, Millipore), O4 (mouse, 1:200, Chemicon) and GFAP (mouse, 1:200, Sigma). After three washes to remove primary antibodies, appropriate secondary antibodies were added (mouse and rabbit) conjugated with FITC, CY3, RPE or biotin (1:200, Jackson ImmunoResearch Laboratories, Inc., and Dako) for 1h at room temperature.

To detect GFP expression, an anti-GFP antibody rabbit serum Alexa 488 conjugated (1:400, Molecular Probes) was used.

The co-localization of GFP signal and cell-specific markers was evaluated by fluorescence microscopy (Zeiss Axiophot) and confirmed by laser confocal scanning microscopy (Leica TCS SP2 AOBS). For an unbiased stereological quantification of cells GFP positive, optical dissectors and random sampling were employed. For this purpose, we considered every tenth coronal sections (20 μm) throughout the entire spinal cord (25 sections per animal).

Cell density evaluation was carried out using the optical dissector protocol. Optical dissectors (100 × 70 × 14 μm) were randomly selected and the number of positive cells in each dissector was defined. The total number of transplanted cells divided by the total volume of optical dissectors determined the cell density.

The volume of tissue per specimen (Vcord) was established by means of the Cavalieri method and, multiplied by the number of donor cells per mm3 (Nv), resulted in the total number of donor cells per specimen (n = Nv × Vcord) [110].

3.8 Evaluation of neuromuscular function and survival

After transplantation, all mice were daily monitored for the typical hallmarks of ALS. All functional analyses were performed by blinded investigators.

Neuromuscular function was determined using an inverted grid hanging test [168]. In this test, mice were placed onto a grid that was gently inverted. The latency to fall was recorded up to 60 seconds. Mortality was scored as the age at death. Animals were sacrificed if were unable of righting themselves within 30s when positioned on their back [110].

3.9 Neuropathological analysis of MNs, axons and NMJs

To determine the number of MNs, paraffin serial sections (12.5 μm) of lumbar spinal cord were Nissl-stained using methylene blue and analyzed using the optical microscope.

For axon counting, spinal cord was soaked in 2.5% glutaraldehyde overnight and post-fixed in 2% osmium tetroxide. Samples were then dehydrated in ethanol and embedded in Epon resin. Toluidine blue staining was performed on semi-thin transverse sections of L4 roots and then examined with light microscopy [110].

The NMJ evaluation was performed on the tibialis anterior (TA) muscle of each mouse. The staining was performed using α-bungarotoxin-555 conjugated (1:500, Life Technologies) and the 2H3 antibody (neurofilament clone 2H3, mouse, 1:100, Developmental Studies Hybridoma Bank)

Muscles were dissected and immunohistochemically processed as described above. Across muscles, we identified a minimum of 50 α-bungarotoxin positive motor end plates and evaluated for overlapping 2H3 positive MN terminal. The 2H3 neurofilaments determine the type of NMJ, which could be categorized in: distinct, when the staining of 2H3 is bright, defined and overlying the endplate; diffuse, if the staining is undefined or partially overlying the endplate; devoid, when there is no 2H3 signal or no overlying the endplate.

3.10 Macro- and micro- gliosis evaluations

Microglial cells and astrocytes quantification was achieved by immunohistochemistry. Sections of the lumbar spinal cord for each mice in each group were processed as described above and stained with primary antibodies: GFAP (mouse, 1:200, Sigma), s100β (rabbit, 1:3000, Abcam) and Iba1 (rabbit, 1:500, Wako).

3.11 Analysis of GSK3β activity

In vitro, the GSK3β activity was evaluated by immunocytochemistry using an anti-P-GSK3β (rabbit, 1:100, Cell Signaling) in iPSC-derived MNs [169].
For the *in vivo* analyses, level of GSK3β protein was quantified by western blot in spinal cord of WT and affected mice.
Frozen spinal cord specimen were homogenized in RIPA-buffer (50 mMTris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktail tablets (Roche). The protein content was measured using Lowry assay. An amount of 30 µg of total protein was boiled in Laemmli buffer for 5 min and separated on an 8% sodium dodecyl sul-fate-polyacrylamide gel electrophoresis SDS-PAGE. After electrophoresis, proteins were transferred on a nitrocellulose membrane, blocked in 5% BSA (for 1 hour at room temperature) and incubated at 4°C overnight with primary antibodies. The following antibodies were used: GSK3β (rabbit, 1:2500, Abcam), Akt (rabbit, 1:1000, Sigma), β-catenin (mouse, 1:500, Santa-Cruz), actin (rabbit, 1:1000, Sigma). The day after, the membranes were incubated with appropriate secondary peroxidase-conjugated antibody (Dako, diluted 1:2700), and signals were detected with an ECL detection kit (Amersham, GE Healthcare).

3.12 Differentiation of human iPSCs into spinal MNs

We applied a multistep differentiation protocol to promote formation of spinal MN phenotype starting from iPSCs [109]. First, iPSCs were differentiated in NSCs using the 10 day protocol described above (DMEM/F12, Neaa, N2 and heparin). Then we added retinoic acid (0.1 μM; Sigma-Aldrich) to induce neural caudalization. To promote ventralization, we added Sonic hedgehog (100-200 ng/ml; R&D Systems) at day 17. For MNs maturation, BDNF (brain-derived neurotrophic factor) and GDNF (glial cell-derived neurotrophic factor) (10 ng/ml; PeproTech) were added at day 24. To enrich the MN population, a centrifugation gradient protocol was used [170]. Cells were fixed after at least 24 hours post plating and stained for the neuronal and MN markers like SMI-32 (mouse, 1:100, Millipore), Islet1/2 (rabbit, 1:200, Millipore), Hb9 (rabbit, 1:200, Millipore), ChAT (Goat, 1:125, Millipore), MAP2 (mouse, 1:100, Sigma). MNs were also transduced with lenti-Hb9::GFP [170].

3.13 Co-culture of MNs and SOD1G93A astrocytes

Murine astrocytes and iPSC-derived MNs were plated in a transwell co-culture assay. In this assay, the porous membrane separates the two compartments allowing only the diffusion of soluble components.

On the bottom chamber of the co-culture, iPSCs-MNs were seeded. Astrocytes were obtained either from newborn spinal cords of SOD1G93A and WT mice [170-172] and were grown in glial medium: DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 1% of penicillin/streptomycin (Life Technologies).

To remove any remaining microglial cells, 2-week-old flasks were maintained in agitation (200 rpm for 6 h) and astrocytes were detached by 0.25% trypsin (Life Technologies) and reseeded at a density of 20.000 cells per cm. To evaluate their neuroprotection ability, NSCs were plated in the upper compartment 48 hours after induction of astrocyte toxicity. Co-cultures were maintained for an additional 3 weeks. To identify MN cells, the Hb9::GFP gene reporter was used [170]. iPSC-derived MNs were also stained for: Islet1/2, Hb9, ChAt, MAP2 and Smi-32 (antibody's information described above) and P-GSK3β (rabbit, 1:100, Cell Signaling).
In order to evaluate the survival of MNs, cell counting was performed using 10 randomly selected fields per well (three wells/condition/experiment in four to five experiments).

3.14 Enzyme-linked immunosorbent assay

Secretion of BDNF, GDNF, NT3 and TGF-α by NSCs was measured in duplicate by ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Brefly, fresh neural medium was added to NSC culture and, 24 hours later, it was collected for the ELISA test. Neural medium was centrifuged and 50 µl of supernatant were added to an ELISA microplate pre-coated with monoclonal antibodies specific for human BDNF, GDNF, NT3 or TGF-α. After 2 hours of incubation, specific enzyme-linked polyclonal antibodies were added to each well and incubated for 2 hours. A substrate solution was added and color develops in proportion to the amount of cytokines bound in the initial step. The stop solution was used to block color development. The color intensity was read at 450 nm within 30 minutes. The standard curve showed a direct correlation between optical density and neurotrophin concentration.

3.15 Analysis of TRPV1 expression and cytotoxicity assay

Conditioned medium experiments and cytotoxicity assay were performed to analyze the transient receptor potential vanilloid subfamily member-1 (TRPV1) on astrocytes. Astrocytes were stained with GFAP (green) and TRPV1 (red). Cells were plated on µ-slide 8-well plates coated with poly-L-lysine (PLL) and treated with NPC-conditioned medium with or without TRPV1 antagonist. As positive control, the ER stress inducer thapsigargin (30 ng/ml) was added. For live-cell ER labelling, ER tracker solution (500 nM) was added to the cells for 30 min at 37 °C. Subsequently, cells were fixed with 4% PFA for 15 min at room temperature. After incubation with NPC-conditioned medium, the relative increase in ER size was quantified by confocal microscopy. As control, non-conditioned media was used and incubation with thapsigargin was set as 100%. For antagonist treatment, the cells were pre-incubated with antagonists for 3 h in control medium. Afterward, the medium was changed with medium containing agonist and antagonist. CytoTox-Fluor Cytotoxicity Assays (Promega) were measured (485 nm/520 nm) with the fluorometer.

3.16 Statistical analysis

For statistical analyses, we used StatsDirect for Windows (version 2.6.4).In all analyses, the null hypothesis was rejected when the p-values were less than 0.05. In culture assays, differences between means were analyzed using the two-tailed Student's t-test. ELISA was evaluated by Student's t-tests. The *in vitro* and *in vivo* neuropathological parameters were analyzed by one-way ANOVA followed by a Tukey post hoc analysis. For survival comparison, Kaplan–Meier survival curve and the log-rank test were used. The functional results were analyzed by an ANOVA two way test for multiple comparisons.

4. RESULTS

4.1 Generation of iPSCs and their differentiation into NSCs

For our *in vitro* and *in vivo* experiments, we used NSCs derived from human differentiated iPSCs. We generated an iPSC line reprogramming fibroblasts obtained from skin biopsies of a control subject (Fig. 5a). For iPSC generation, we used a non-viral and non-integrating method based on electroporation of three plasmids encoding for the key transcription factors of stem cells: OCT4, SOX2, c-MYC, NANOG, KLF4 and LIN28 (Fig. 5b).

During proliferation, cells progressively lost these three plasmids, leading to the generation of iPSCs free from vectors and exogenous sequences. The iPSCs showed the typical embryonic stem cell morphology, growing as colonies in adhesion and forming EBs when re-suspended in low attachment conditions. All iPSCs expressed markers of pluripotency such as SSEA4, TRA1-60, TRA1-81, SOX2 and OCT4 (Fig. 5c).

iPSCs maintained euploid karyotipe and could differentiate into all three germ layers (ectoderm, mesoderm and endoderm) both *in vitro* and *in vivo*, forming teratomas after transplantation into immunodeficient mice (data not shown).

Once we generated and fully characterized our iPSC line, we differentiated it into NSCs using a multistage protocol optimized to allow the generation of a neuronal fate. This protocol allowed us to generate a high percentage of neuroepitelial cells positive for NSC and precursor markers such as PAX6, SOX2 and Nestin (>90%).

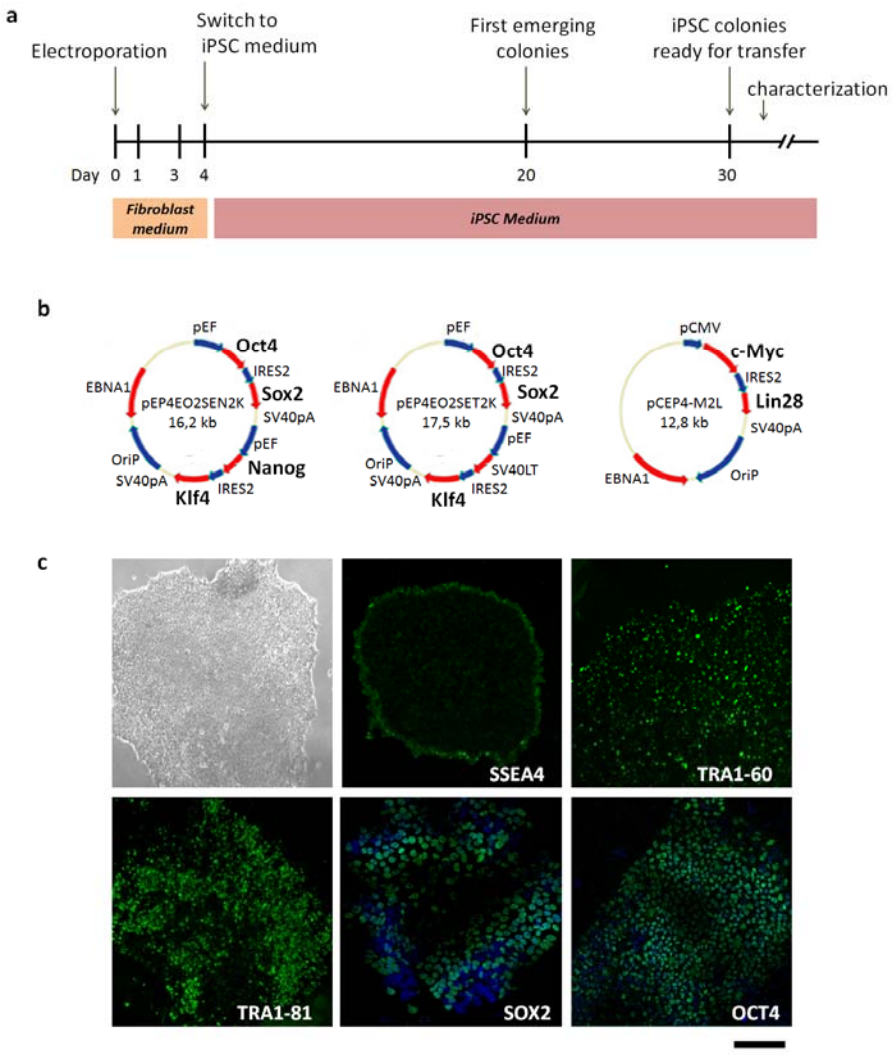

Fig. 5 Generation of human iPSCs.

(**a**) Schematic representation of the non-viral, non-integrating reprogramming method used for iPSC generation starting from control skin fibroblasts. (**b**) Maps of episomal vectors encoding for pluripotency transcription factors such as OCT4, SOX2, c-MYC, NANOG, KLF4 and LIN28. (**c**) Colonies of iPSCs in adhesion conditions. In the first panel, a colony of iPSCs in bright field; in other panels, immunocytochemistry characterization of this control iPSC line, which expressed the typical markers of pluripotency, SSEA4, TRA1-60, TRA1-81, SOX2 and OCT4 (in green). Scale bar: 75 μm (SOX2 65 μm). In blue, nuclei stained with DAPI.

4.2 Selection of LeX+CXCR4+β1+ NSCs and characterization

After differentiation of iPSCs into NSCs via EBs (Fig. 6a), we employed a combination of immunomagnetic (MACS) and fluorescence-activated cell sorting (FACS) analysis in order to select the subpopulation of NSCs positive for Lewis X, CXCR4, and β1 integrin (Fig. 6).

Since preliminary analyses of these three markers revealed a relative low percentage of cells positive for CXCR4, we enriched our NSC line for the CXCR4+ population using MACS. Enriched cells were later analyzed by FACS, which showed an increased percentage (81.4%) of CXCR4+ NSCs (Fig. 6b).

After plating and expansion, CXCR4+ cells were further selected by FACS for β1 integrin (or CD29), LewisX (LeX or CD15) and CXCR4 positivity. FACS re-analysis showed an enrichment of NSCs positive for the three markers (Fig. 6c).

After LeX+CXCR4+β1+ NSC selection, we further expanded them in neuronal media supplemented with fibroblast growth factor (FGF)-2 in adherent conditions and we fully characterized them (Fig. 7).

Immunocytochemistry confirmed the expression of the three markers (Fig. 7a). The expression of the typical antigens of NSCs, such as PAX6, SOX2 and Nestin (Fig. 7b) confirmed the neural property of this subpopulation. The cells maintained also their multipotency and were able to differentiate into neurons, astrocytes, and oligodendrocytes (Fig. 7c).

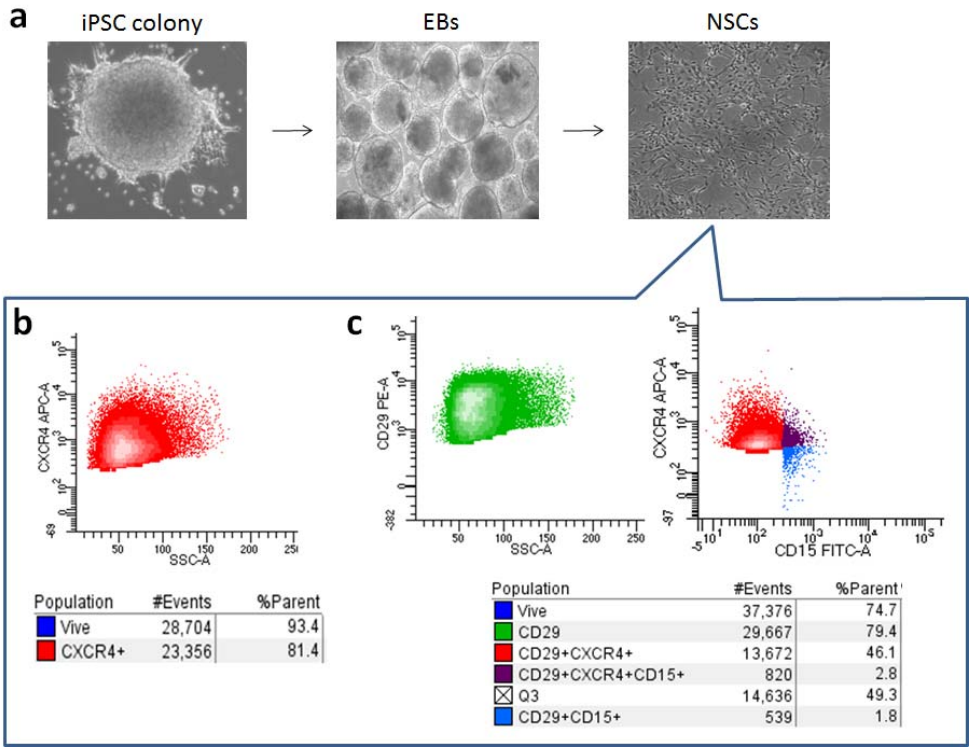

Fig. 6 NSC generation and selection for Lewis X, CXCR4 and β1 integrin.

(**a**) NSC generation: iPSC colonies were detached and maintained in suspension in order to form EBs, which were treated with neuronal medium. The dissociation of EBs allowed NSC formation in appropriate neuronal media. (**b**) FACS analyses revealed a high percentage of CXCR4+ NSCs after MACS sorting (81.4% of CXCR4+ NSCs). (**c**) Selection of NSCs for their positivity for the three markers: Lewis X (or CD15), CXCR4 and β1 integrin (or CD29).

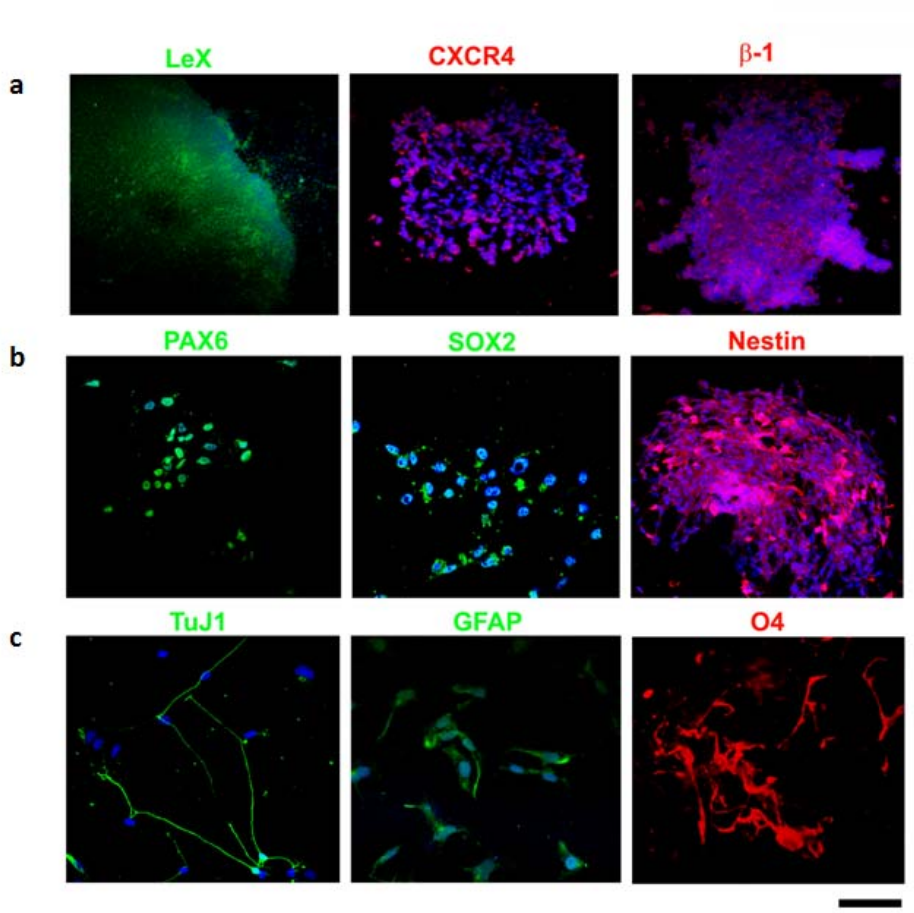

Fig. 7 Characterization of LeX+CXCR4+β1+ NSCs.

(**a**) Immunocytochemistry of the FACS selected triple positive subpopulation of NSCs for the three markers, Lewis X (in green), CXCR4 and β1 integrin (in red). (**b**) NSCs expressed the marker of NSCs like PAX6, SOX2 (in green) and Nestin (in red) and (**c**) could differentiate into neurons (TuJ+, in green), astrocytes (GFAP+, in green) and oligodendrocytes (O4+, in red). Nuclei are stained in blue with DAPI. Scale bar: 100 μm LeX; 70 μm CXCR4; 75 μm β1; 65 μm PAX6; 50 μm SOX2; 70 μm Nestin; 50 μm in the (c) panel.

4.3 LeX+CXCR4+β1+ NSC transplantation in SOD1G93A mice

We evaluated the ability of LeX+CXCR4+β1+ NSCs to engraft the spinal cord and to migrate through the host nervous system parenchyma by tracking them with the GFP reporter.

One million of cells were administered into the cerebrospinal fluid (CSF) by intrathecal injection into the SOD1G93A transgenic mice, the ALS model that carries high copy number of human SOD1 gene with the G93A mutation (Fig. 8a). One month after administration, we observed a relevant engraftment of donor cells GFP positive in the host parenchyma (Fig. 8b). Some transplanted cells were able to differentiate into mature neurons and MNs, as revealed by co-localization of TuJ1 or Smi32 and GFP reporter respectively (Fig. 8 b, c).

Analyzing the entire spinal cord, we observed that the major part of GFP+ NSCs was localized in the lumbar spinal cord closed to the site of injection. However, a relevant number of cells were detected also in both cervical and thoracic spinal cord (Fig. 8d). In particular, we observed clusters of NSCs in the anterior horns area of the spinal cord, where MNs are actively degenerated. After transplantation, we also characterized the phenotypic fate of donor cells. We observed that the vast majority of transplanted cells maintained neural stem phenotype, as demonstrated by a consistent portion of GFP positive cells expressing Nestin, the NSC marker (Fig. 8e). Only a small percentage of donor cells differentiated into mature neurons and microglial cells. Among donor cells that acquired glial commitment, we observed a more relevant differentiation of NSCs into astrocytes compared to oligodendrocytes, which were less than 5% (Fig. 8e).

Transplantation of NSCs did not cause adverse effects like tumors or abnormal cell proliferation. No inflammation was observed in treated mice.

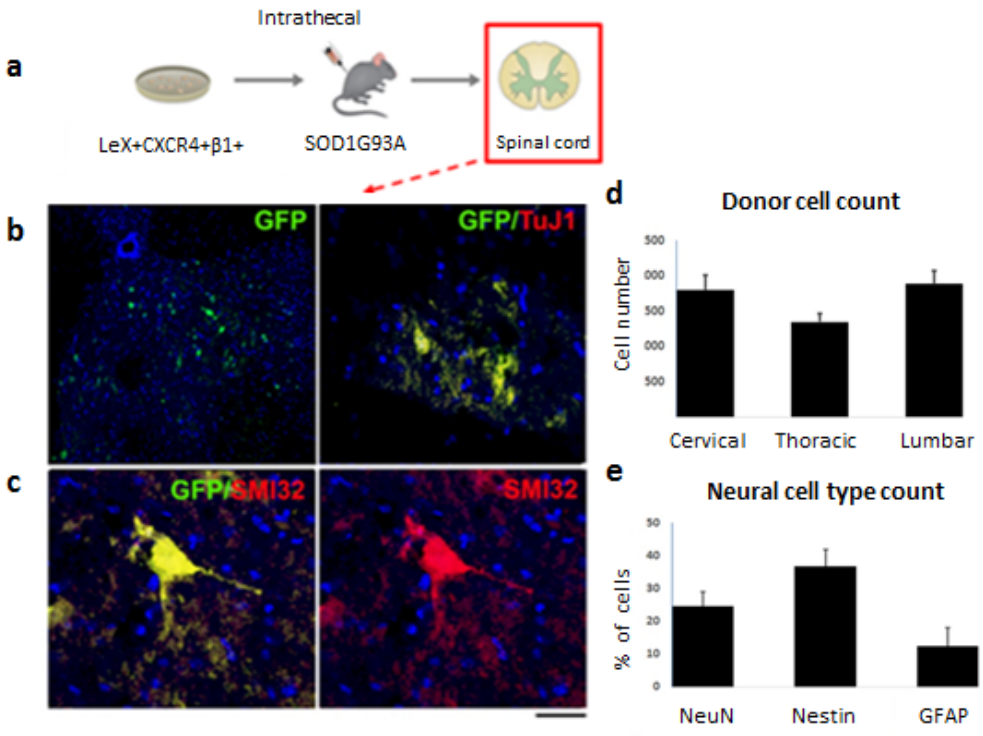

Fig. 8 Transplantation of LeX+CXCR4+β1+ NSCs into ALS mice.

(**a**) Representative illustration of the *in vivo* experiments: selected NSCs were intrathecally administered in the ALS mouse model (SOD1G93A) at 90 days of age. One month after, spinal cords of treated mice were analyzed. (**b**) Immunohistochemistry revealed that GFP+ NSCs (in green) were able to differentiate into neurons (TuJ1+, in red) in the host spinal cord. (**c**) Donor cells GFP+ were also able to differentiate into MNs as shown by their positivity for Smi32 (in red). (**d**) Quantitative analysis of GFP+ NSCs in the spinal cord revealed transplanted cells in the entire spinal cord. In fact, GFP+ NSCs were detected in cervical, thoracic and lumbar spinal cord. (**e**) Quantitative analysis of the phenotypic fate of engrafted cells showed that a significant portion maintained the neural stem/precursor phenotype (nestin) and a small fraction differentiated into mature neuronal (NeuN) and glial (GFAP) phenotypes. Data are shown as mean ± SD. Nuclei are stained with DAPI (in blue). Scale bars: 150 μm in (b) panel and 50 μm in (c) panel.

4.4 LeX+CXCR4+β1+ NSCs protect MNs, axons and NMJs

The typical hallmark of ALS consists in a progressive degeneration of MNs. For this reason, we performed quantification of MNs in SOD1G93A mice at 120 days of age of NSC-treated mice and controls, which were treated with fibroblasts or saline solution (vehicle). The results of NSC transplantation consisted in a significantly reduction of MN death compared to vehicle-treated SOD1G93A mice ($P < 0.05$) (Fig. 9 a, c). Furthermore, we observed a preservation of axons after NSC treatment. The analysis of lumbar L4 ventral roots, revealed an higher number and size of axons in NSC-treated SOD1G93A mice at 120 days ($P < 0.05$) (Fig. 9 b, d).

The preservation of MNs and their axons had a significant effect on NMJs. We observed a delay in term of NMJs denervation in NSC-treated SOD1G93A mice respect to animals treated with vehicle (Fig. 9 e, f). Moreover, NSC-treated mice showed an almost preserved number of intact pretzel-shaped NMJs (Fig. 9g). In NSC-treated mice, the fully innervated NMJs were 65.2 ± 5.7 % compared to 21.8 ± 4.9 % in controls ($P < 0.01$) (Fig. 9g). Also, we noticed a relevant reduction in term of completely or partially denervated NMJs compared to SOD1G93A mice treated with vehicle.

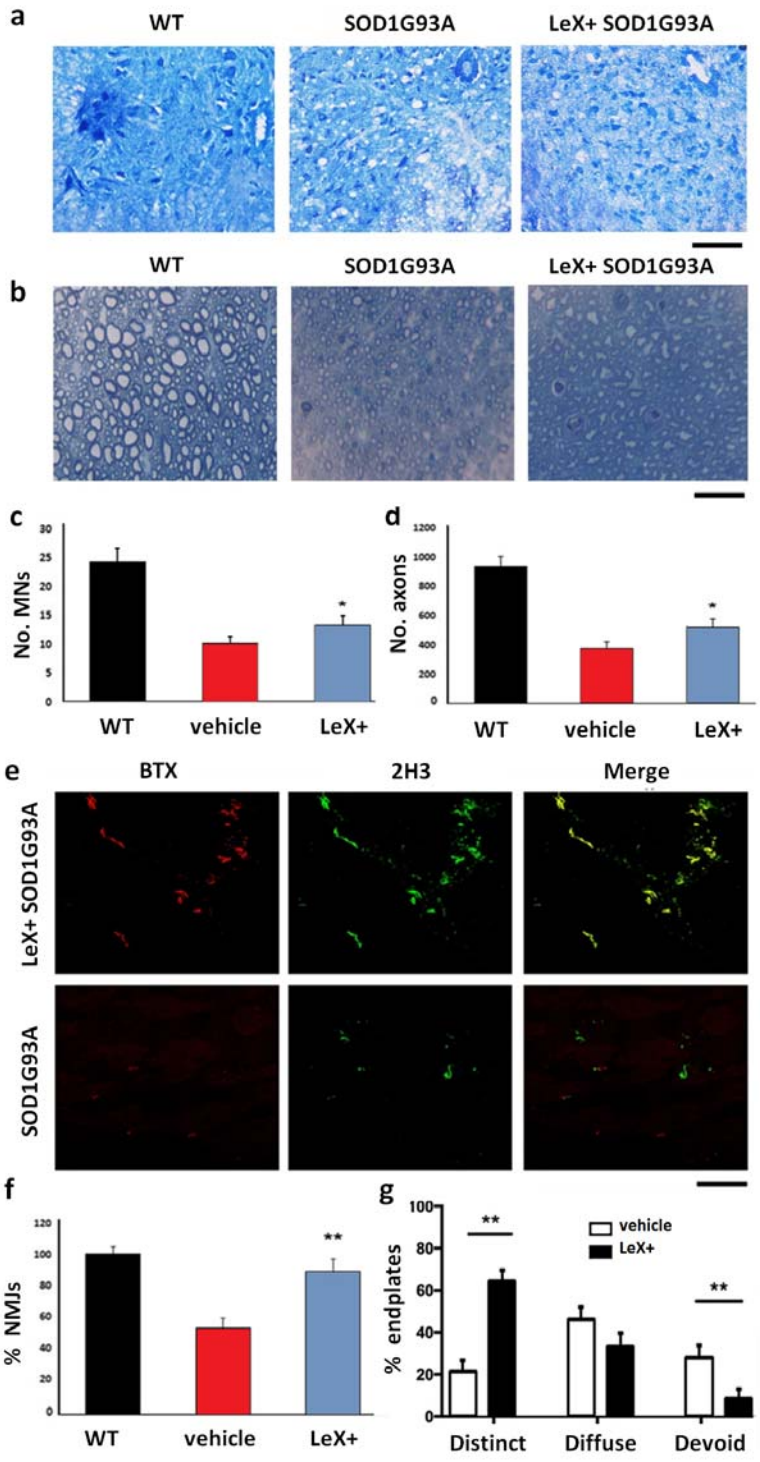
a
WT
SOD1G93A
LeX+ SOD1G93A
b
WT
SOD1G93A
LeX+ SOD1G93A
c
No. MNs
WT
vehicle
LeX+
d
No. axons
WT
vehicle
LeX+
e
BTX
2H3
Merge
LeX+ SOD1G93A
SOD1G93A
f
% NMJs
WT
vehicle
LeX+
g
% endplates
vehicle
LeX+
Distinct
Diffuse
Devoid

Fig. 9 Transplantation of LeX+CXCR4+β1+ NSCs protects MNs, axon and NMJs.

(**a**) Nissl-staining on spinal cords of WT and affected mice treated with vehicle or NSCs. (**b**) Toluidine blue staining performed on spinal cords of all three animal groups. (**c**) Quantitative analyses of MNs in the lumbar spinal cord at 120 days of age. ALS mice treated with NSCs showed an increased number of MNs compared to controls. Data are mean ± SD (**P* < 0.05, ANOVA). (**d**) Axon quantification showed a higher number in NSC-treated mice compared to controls. We considered 6 animals per group at 120 day of age. Data are mean ± SD (**P* < 0.05, ANOVA). (**e**) Staining on anterior tibialis (TA) muscles with post-synaptic α-bungarotoxin (in red) and pre-synaptic 2H3 (in green). (**f**) Quantitative analyses of NMJs in the TA muscles showed an increased percentage of NMJs in NSC-treated mice (***P* < 0.01, ANOVA, n=6, data are mean ± SD). (**g**) Qualitative analyses of NMJs revealed an increased number of distinct NMJs compared to controls and a reduction of devoid NMJs (***P* < 0.01, ANOVA, n=6, values are mean ± SD). Scale bar: (a) 100 μm; (b) 75 μm; (e) 50 μm.

4.5 NSC transplantation improves ALS phenotype and survival

We investigated the therapeutic potential of LeX+CXCR4+β1+ NSCs on muscular function and survival in SOD1G93A mice.

Mice treated with NSCs showed an increased muscle strength compared to controls, as demonstrated by the inverted grid assay ($P < 0.05$, Fig. 10a). SOD1G93A mice treated with vehicle were progressively weaker and unable to move with the disease progression. Vice versa, SOD1G93A mice treated with NSCs appeared healthier and were able to walk and explore the cage. These evidences support the positive effects of NSC transplantation on neuromuscular phenotype.

Remarkably, we observed a significant increase in the survival of NSC-treated mice. In fact, the average of life span was 165 ± 5 days for NSCs-treated animals (n=12) and 142 ± 11 days (n=15) for vehicle-treated mice (Chi-square for equivalence of death rates= 14.32, $P = 0.0002$, Kaplan–Meier log rank test for survival) (Fig. 10b).

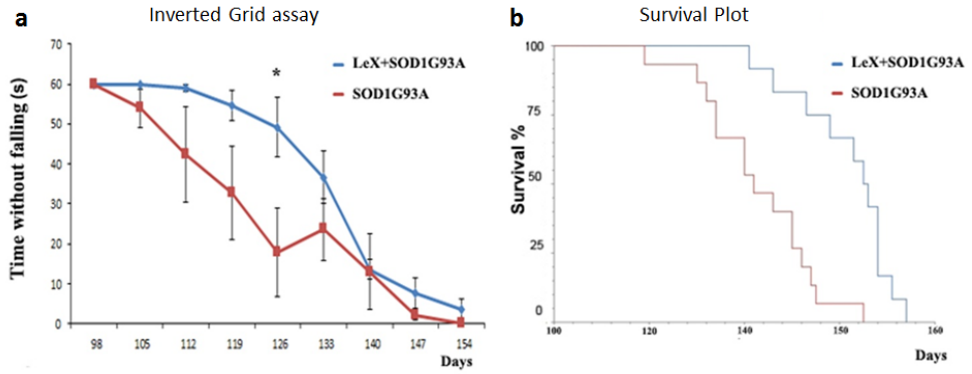

Fig. 10 Improvement of neuromuscular function and survival in ALS mice treated with LeX+CXCR4+β1+ NSCs.

(**a**) NSC transplantation determined amelioration of neuromuscular function as demonstrated by inverted grid hanging assay showing an improved ability of NSC-treated mice (*$P < 0.05$, ANOVA). (**b**) Kaplan-Meyer survival curves for ALS mice treated with NSCs or vehicle. The survival was increased in NSC transplanted mice (P = 0.0002, log-rank test).

4.6 LeX+CXCR4+β1+ NSCs reduce macro- and micro- gliosis

During disease progression, reactive astrocytes and microglial cells accumulate in the spinal cord of ALS patients and animal models. These findings support the relevance of non-autonomous component in ALS pathogenesis [82]. For this reason, we investigated whether NSCs were able to reduce astrocytosis, the abnormal proliferation of toxic astrocytes. In particular, we detected a significant reduction of reactive astrocytosis after NSCs transplantation. Indeed, GFAP and s100β staining revealed a significantly reduction of astrocytes in the spinal cord parenchyma of NSC-treated mice compared to vehicle-treated SOD1G93A mice ($P < 0.05$) (Fig. 11 a, c). We also observed a significant reduction in microglia cells (Iba1+) in the spinal cord of NSC-treated SOD1G93A respect to controls ($P < 0.05$) (Fig. 11 b, d).

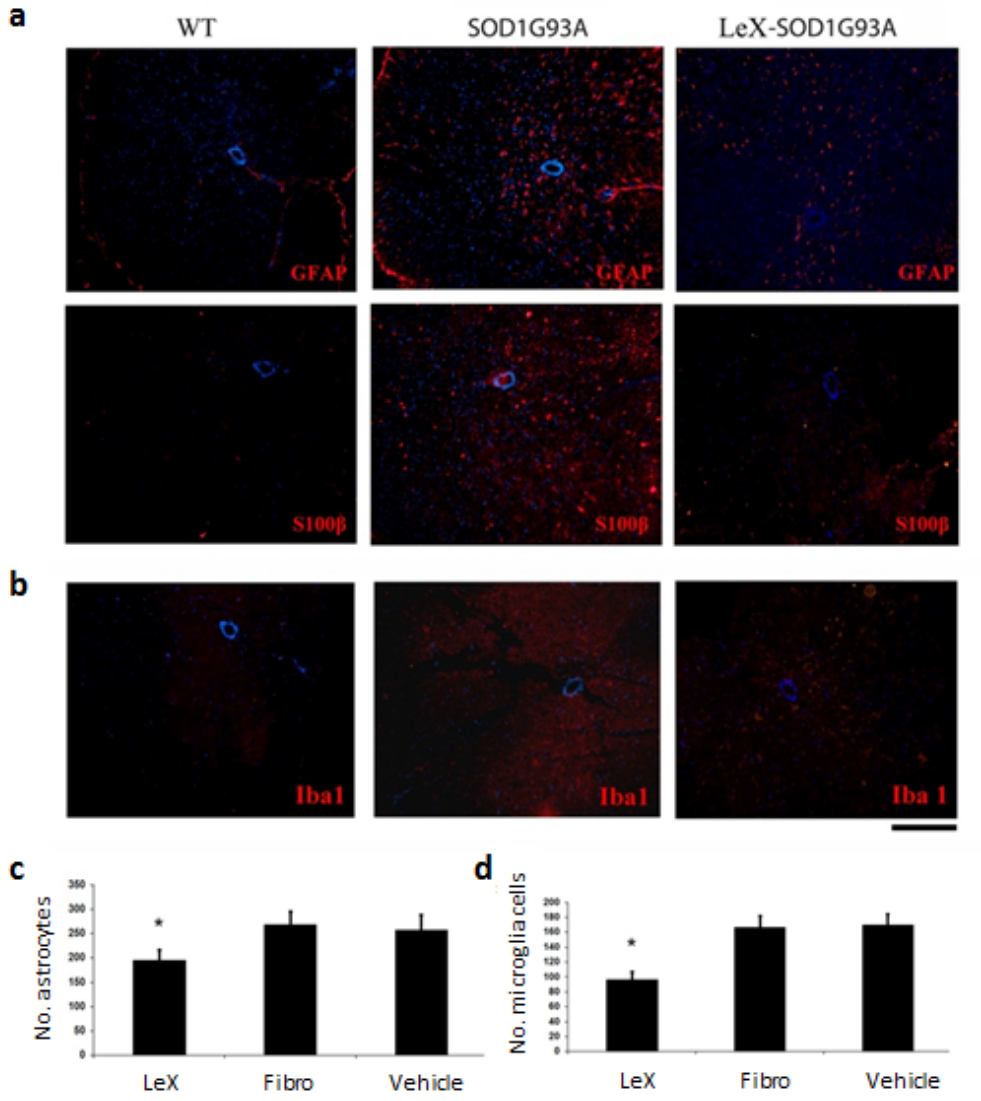

Fig. 11 Macro- and micro- gliosis analysis.

(**a**) Immunostaining with GFAP and s100β for macrogliosis and (**b**) with Iba1 for microgliosis in all three animal groups (WT, SOD1G93A mice treated with vehicle, SOD1G93A mice NSC-treated). Nuclei are in blue stained with DAPI. Glial markers are in red. (**c**) Quantification of astrocytes in spinal cord revealed a reduction of astrocytosis in NSC-treated mice. (**d**) Number of microglial cells was diminished in the spinal cord of NSC-treated mice. All data are shown as mean ± SD.* $P < 0.05$, ANOVA. Scale bar: 150 µm.

4.7 NSCs exert beneficial effects reducing GSK3β

It has been demonstrated that abnormal increase in levels and activity of GSK3β, a serine/threonine protein kinase, are associated with MN death [93, 169] and that its inhibition protects MNs from degeneration [173-175]. In order to investigate the possible molecular mechanisms by which LeX+CXCR4+β1+ NSCs exert their beneficial effects in ALS mice, we analyzed level of GK3β kinase in spinal cord of our cohort of mice. Western blot analyses revealed an increase of 2-folds in GSK3β expression in ALS mice compared to WT animals. Despite treatment with NSCs determined a reduction of GSK3β protein in spinal cord of affected mice, this reduction is not statistically significant (Fig. 12).

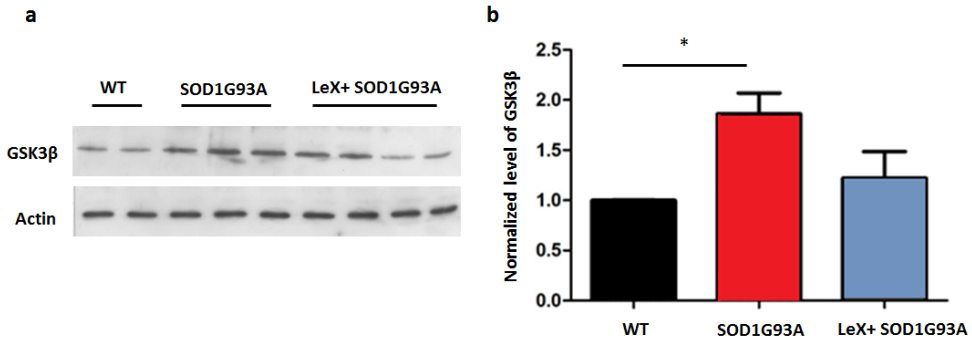

Fig. 12 Levels of GSK3β in spinal cord.

(**a**) Western blots analysis of GSK3β and β-actin in the spinal cord of all three groups of mice. (**b**) Densitometry analyses of the GSK3β expression in spinal cord of WT and SOD1G93A mice treated with vehicle or LeX+CXCR4+β1+ NSCs. When normalized for β-actin protein expression, there was significant difference in GSK3β expression between WT and ALS mice (* $P < 0.05$). However, no difference occurs between ALS mice treated with vehicle or NSCs.

4.8 LeX+CXCR4+β1+ NSCs protect human ALS MNs in vitro

To investigate whether NSCs exert a therapeutic effect also in a human *in vitro* model of ALS, we employed a co-culture assay using iPSC-derived MNs, murine ALS astrocytes and LeX+CXCR4+β1+ NSCs (Fig. 13a). First of all, to generate spinal MNs from control and ALS iPSC lines, we used a multistep protocol based on RA and Shh [109]. After 4 weeks of differentiation conditions, cells expressed the typical markers of MN such as Smi32 and Hb9 (Fig. 13b) and also pan-neuronal markers such as TuJ1, Neurofilament, and MAP2 were observed (data not shown). Since the protocol of differentiation yielded a mixed cell population, we enriched MN population applying a physical strategy based on gradient centrifugation. Then, iPSC-derived MNs from both control subjects and ALS patients were co-cultured with toxic ALS astrocytes derived from SOD1G93A mice. Before plating with ALS astrocytes, both control and affected iPSC-derived MNs were visualized by a lenti-Hb9::eGFP construct, appeared control and showed elongated processes (Fig. 13c). On the contrary, when plated with ALS toxic astrocytes, iPSC-derived MNs progressively degenerate after 20-23 days of culture (Fig. 13 c, d). In particular, MNs derived from both familial and sporadic ALS patients were more sensitive to the toxicity of ALS astrocytes compared to control MNs ($P < 0.01$, Fig. 13d). However, when NSCs were added to the co-culture, we observed a significant protection of MNs. In fact, when exposed to LeX+CXCR4+β1+ NSCs, iPSC-derived MNs were significantly spared ($P < 0.01$) (Fig. 13d). Therefore, our findings demonstrate that NSCs can protect affected MNs from degeneration in an ALS-like toxicity system.

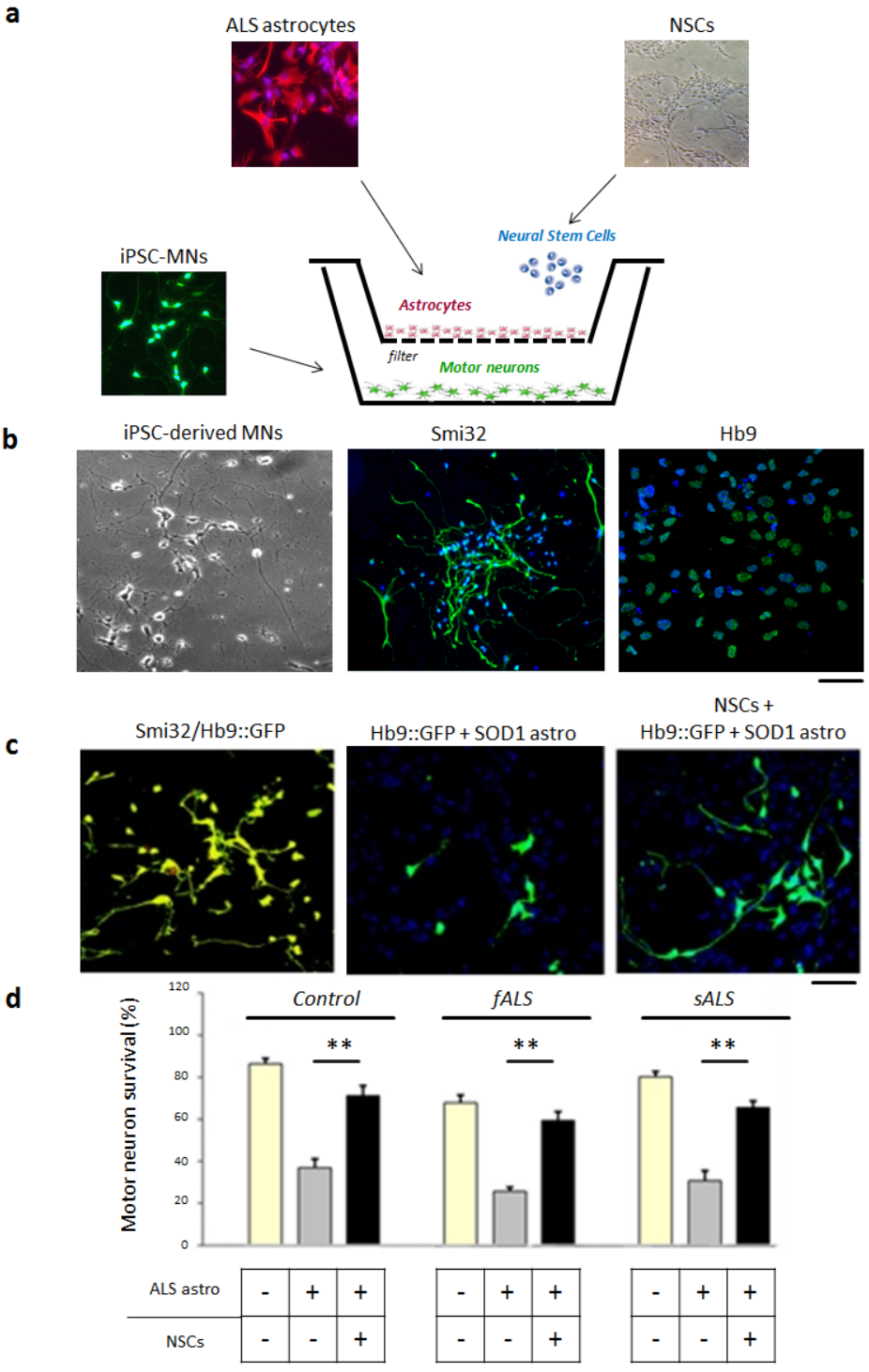
a
ALS astrocytes
NSCs
iPSC-MNs
Neural Stem Cells
Astrocytes
filter
Motor neurons
b
iPSC-derived MNs
Smi32
Hb9
c
Smi32/Hb9::GFP
Hb9::GFP + SOD1 astro
NSCs +
Hb9::GFP + SOD1 astro
d
Motor neuron survival (%)
120
100
80
60
40
20
0
Control
fALS
sALS
**
**
**
ALS astro
NSCs
- + +
- - +
- + +
- - +
- + +
- - +

Fig. 13 LeX+CXCR4+β1+ NSCs protect human MNs *in vitro*.

(**a**) Schematic representation of co-culture assay, which consisted in human control and ALS iPSC-derived MNs (in green) and murine SOD1G93A astrocytes (in red). In this type of co-culture, also selected NSCs were plated (in bright field). (**b**) MNs derived from differentiation of human iPSCs. In the first panel, iPSC-MNs in bright field, other panels showed the expression of the typical markers of MNs like Smi32 and Hb9 (in green). (**c**) Before plating MNs with astrocytes in our co-culture system, iPSC-derived MNs were labeled with the construct lenti-Hb9::eGFP and stained with Smi32 in red (first panel in c). As showed in the second panel, the number of iPSC-derived MNs was reduced when plated with SOD1G93A astrocytes. However, the presence of NSCs in the co-culture determined a preservation of MNs exposed to astrocytes toxicity (last panel in c). Nuclei stained in blue with DAPI. (**d**) MN quantification in different conditions: MNs plated with control or ALS astrocytes and with or without NSCs. Quantification revealed a decreased number of MNs after co-culture with SOD1G93A astrocytes (***P* < 0.01, ANOVA), however the presence of NSCs improved MNs survival (***P* < 0.01, ANOVA). Data are means ± SEM from 5 experiments in triplicate. Scale bar: 50 μm (Hb9 70 μm).

4.9 NSCs protect human MNs by inhibiting GSK3β activity

In this human *in vitro* ALS model, we analyzed the putative mechanisms of neuroprotection induced by NSCs. Since increased levels of GSK3β were found in spinal cord of ALS mice, we analyzed the role of this kinase also *in vitro*. Abnormal levels or hyper-activation of GSK3β determines death of MNs. Based of these findings, we analyzed GSK3β activity in iPSC-derived MNs. Inactivation of GSK3β is determined by a specific phosphorylation in Ser9. For this reason, we evaluated Ser9-site specific phosphorylation of GSK3β (GSK-3PSe9) in iPSC-MNs from ALS and control subjects. Anti-Phospho GSK3β immunocytochemistry revealed that levels of GSK-3PSe9 phosphorylation were reduced in ALS MNs compared to control MNs. However, when exposed to NSCs, affected MNs showed higher levels of GSK-3PSe9 ($P < 0.01$). This data indicate that NSC treatment determined an inactivation of GSK3β in ALS MNs, protecting them from degeneration (Fig.14).

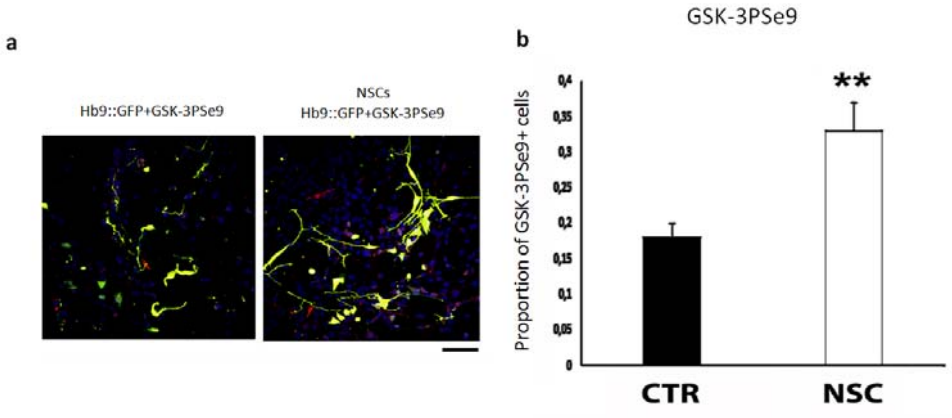

Fig. 14 NSCs increased phosphorylation of GSKβ in human MNs.

(**a**) Staining of Ser9 phosphorylation in the GSK3β enzyme (red signal), in absence (left) or presence (right) of NSCs. In blue, nuclei stained with DAPI. (**b**) Quantitative analysis of GSK-3PSe9 showed a significant difference in the phosphorylation level (** $P < 0.01$). MNs were labeled with Hb9-GFP (green). Scale bar: 75μm.

4.10 NSCs protect MNs by producing neurotrophic factors

In neurodegenerative diseases like ALS, NSCs can be potentially therapeutic through multiple mechanisms, which include not only neuronal replacement but also bystander effects such as the production and delivery of neurotrophic factors. In fact, we and other groups observed that NSCs induce neuroprotection thanks to their ability to deliver neuroprotective molecules [110, 165, 176]. Here, we noticed that NSCs released a wide spectrum of neurotrophic factors and molecules that promote neurite outgrowth. In particular, our subpopulation of NSCs produced glial cell-derived growth factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and transforming growth factor alpha (TGF-α) (versus human fibroblasts; $P < 0.001$) (Fig. 15).

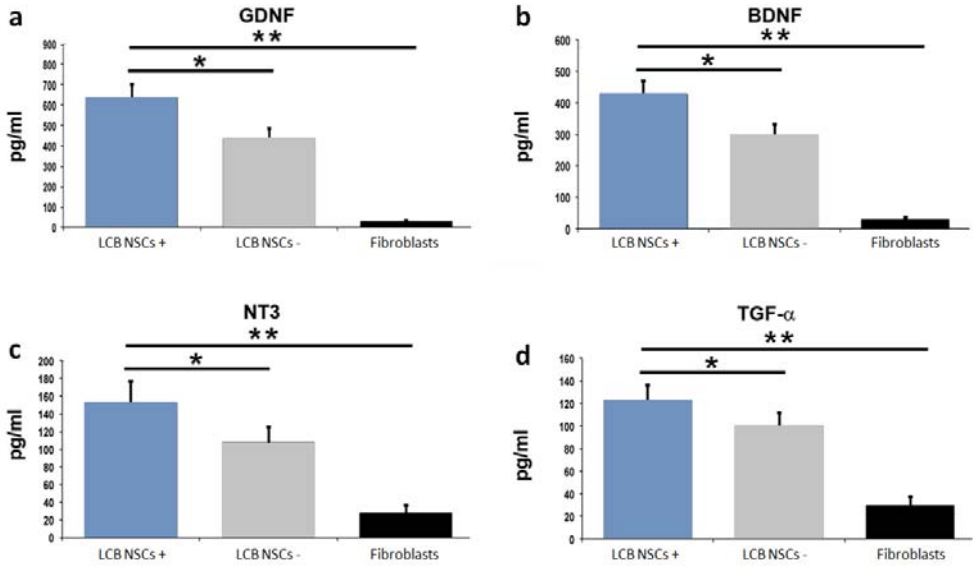

Fig. 15 NSCs produce high levels of neurotrophic factors.

(**a-d**) ELISA test showed that LeX+CXCR4+β1+ NSCs (LCB NSCs+) released higher level of neurotrophic factors, such as GDNF, BDNF, NT3 and TGF-α, compared to NSCs negative for the three markers (LCB NSCs-) and fibroblasts. All data are mean ± SD (* $P < 0.05$; ** $P < 0.01$).

4.11 NSCs inhibit ALS astrocytes proliferation activating TRPV1

We evaluated the effect of LeX+CXCR4+β1+ NSCs on astrocytes proliferation (Fig. 16).

We already showed that SOD1G93A astrocytes exposed to molecules secreted by human ALDH NSCs significantly diminished their vitality [110]. In particular, astrocytes proliferation was reduced by endovanilloids secreted by NSCs that bind the TRPV1 receptor on astrocytes. Thus, we hypothesized a suppressor function on ALS toxic astrocytes of the endovanilloids produced by NSCs [110].

Here, we investigated if this event occurs also with LeX+CXCR4+β1+ NSCs. We demonstrated that astrocytes derived from SOD1G93A mice were TRPV1 positive (Fig. 16a) and the addition of LeX+CXCR4+β1+ NSCs in culture induced astrocytes death (Fig. 16b). Selective inhibitors of TRPV1, like iodoresiniferatoxin (I-RTX) or capsazepine (CZP), reduced NSC cytotoxicity on ALS astrocytes (Fig. 16c). These data show that LeX+CXCR4+β1+ NSCs cause inhibition of ALS toxicity by activating TRPV1.

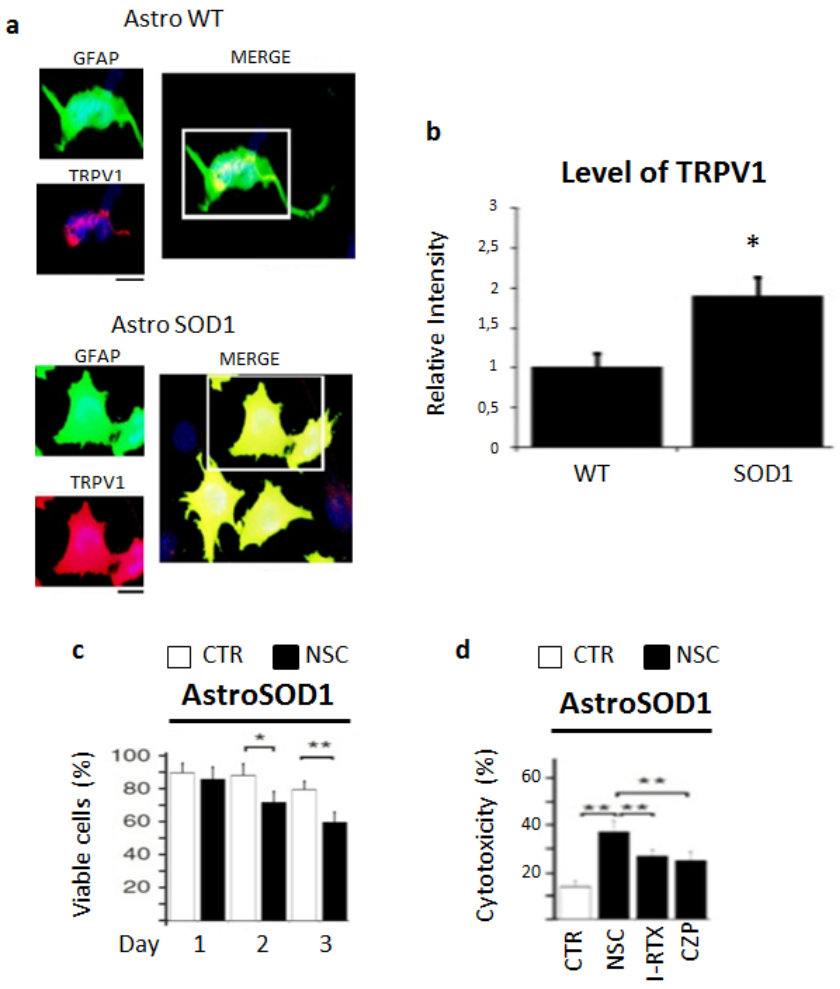

Fig. 16 LeX+CXCR4+β1+ NSCs inhibit astrocytes proliferation via TRPV1 activation. (**a**) Expression of GFAP (green) and TRPV1 (red) in WT astrocytes (upper panel) and in SOD1G93A astrocytes (lower panel). In blue, the nuclei stained with DAPI. Scale bars: 30 μm on the left and 10 μm in the right image with higher magnification. (**b**) Level of TRPV1 was higher in SOD1G93A astrocytes respect to WT astrocytes as quantified in the graph with relative fluorescence intensity (* $P < 0.05$). (**c**) The survival of SOD1G93A astrocytes was reduced when exposed to LeX+CXCR4+β1+ NSC–conditioned medium. (**d**) Inhibition of TRPV1 with I-RTX and CZP determined a reduction of the LeX+CXCR4+β1+ NSCs cytotoxicity on astrocytes, indicating that the beneficial action of LeX+CXCR4+β1+ NSCs is mediated by activation of TRPV1. All results are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$; Student's *t*-test.

5. DISCUSSION

ALS, also known as Lou Gehrig's disease, is a neurodegenerative disorder that affects MNs, the nerve cells involved in the transmission of the electrical impulses from brain to the muscles [1]. In ALS, MNs in the spinal cord, brainstem and cortex undergo to a progressive degeneration and death, determining a loss of the capacity to initiate and control voluntary movements. The consequence of MN degeneration is a progressive paralysis and muscle atrophy. Despite muscle weakness and paralysis are universally experienced, the onset of ALS is variable and could starts in muscles that control legs, feet, arms, hands, speech or swallowing. Among MN diseases, ALS is the most common and severe form in adults; in fact, ALS is particularly aggressive determining death of patients in 3-5 years from the onset of the first symptoms [4]. Currently, there is no effective cure for ALS beyond supportive care and Riluzole, which extends median survival only by about 2 to 3 months.

Because causes and mechanisms involved in ALS are largely unknown, the way to find a cure is particularly difficult. Indeed, ALS is a complex disorder involving an intricate combination of events including genetic, environmental and lifestyle factors [17]. Moreover, a wide spectrum of molecular and cellular mechanisms has been proposed to mediate the progressive MN degeneration in ALS. Besides, to complicate matters further, different cell types contribute to the development of this pathology, that include beside MNs, other neurons, astrocytes, microglia and oligodendrocytes.

Given the multifaceted nature of this neurodegenerative disorder, the therapeutic strategies that targeted only one specific pathogenetic disease aspect likely will have minimal impact on ALS phenotype [101]. On the contrary, approaches that affect many pathophysiological mechanisms at the same time could have a better efficiency. For this reason, stem cell transplantation has recently become an attractive option thanks to its multiple actions that can influence simultaneously many different pathogenetic events. In fact, although the initially proposed mechanism was cell replacement, now it is clear that stem cells may also provide a large number of benefits by modulating the micro-environment, leading to a reduction of inflammation and protecting MNs and neuronal circuitry [148].

Among the different stem cell sources, NSCs are particularly appropriate for ALS treatment due to their capability to primarily differentiate into neuronal and glial cells, providing a valuable life-long source of nervous cells. Moreover, since NSCs are more specialized and differentiated than embryonic stem cells, the risk of forming tumors after transplantation is reduced. The positive effects of NSC transplantation have been already demonstrated in pre-clinical experiments and it is now being explored in clinical trials. In fact, when transplanted into the spinal cords of ALS animal models, NSCs derived from embryonic stem cells or iPSCs determined an effectively improvement in term of survival and pathological phenotype [110, 165]. Besides, a phase I clinical studies revealed that NSC transplantation via intraspinal cord injections in ALS patients is safe and also seems to partially reduce disease progression, even if the therapeutic effect was not principal aim of the trial [166, 167].

In our *in vitro* and *in vivo* experiments, we used a control cell line of NSCs derived from iPSC differentiation. Since iPSCs are directly derived from adult tissues, they not only bypass ethical issue of the embryo manipulation but are also they are patient-specifics, meaning that they can potentially reproduce the ALS features *in vitro*. For these reasons, the iPSC technology is promising for both cell transplantation and for modeling human pathologies *in vitro*. In this study, the iPSCs were generated by reprogramming differentiated adult cells to a state of pluripotency, through the expression of transcription factors using a non-viral and non-integrating technique. We reprogrammed fibroblasts derived from skin biopsies of a control subject performing electroporation of plasmids, which encode for the key transcription factors of stem cells such as OCT4, SOX2, c-MYC, NANOG, KLF4, and LIN28 [104, 109-111]. We isolated a control iPSCs line that showed the embryonic stem cell morphology and expressed the typical markers of pluripotency like SSEA 3 and 4, TRA1-81, TRA1-60, OCT4 and SOX2. In addition, further analyses revealed that iPSCs maintained euploid karyotype and their ability to differentiate into all three germ layers both *in vitro* and *in vivo*.

Once generated and characterized, iPSCs were differentiated into NSCs using a multistage protocol, which consisted in EB formation and treatment with small molecules to induce neuralization. This technique was optimized in order to generate a high percentage of neuronal fate cells; in fact, more than 90% of cells were positive for PAX6, a NSC marker [110].

Since the efficiency of cell transplantation can be limited by a reduced ability of cells to migrate and reach the affected areas, we selected a subpopulation of NSCs in order to increase their ability to engraft and migrate through the nervous system parenchyma, to protect degenerating MNs and to improve ALS phenotype. In particular, we sorted NSCs for the presence of three markers: Lewis X, CXCR4 and β1 integrin.

These three markers were crucial for migration and engraftment of transplanted NSCs for the following reasons:

- *Lewis X*, also called CD15 or SSEA-1, is an adhesion molecule expressed on cell surface and it is one of the most important ligand of receptors present on endothelial cells [177]. It is also a marker of pluripotent stem cells and plays a relevant role in cell migration and adhesion in the pre-implantation embryo [178]. In particular, Lewis X mediates the extravasation of cells positive for this marker and increases the ability of cells to migrate through the spinal cord.
- *CXCR4* is a chemokine receptor, which increases the sensitivity of the cells to be recruited by the host diseased spinal cord that produces chemoattractant cytokines. During embryogenesis, CXCR4 is expressed by newly generated neurons. In adult life, it plays a role in neuronal guidance. It also seems to be involved in the adhesion phase of human embryonic implantation. Cells positive for CXCR4 could migrate from cerebrospinal fluid (CSF), that bathes the entire central nervous system, to spinal cord thanks to proinflammatory cytokines, which are produced by injured central nervous system (CNS). Among molecules produced by sites of damage, the SDF1 alpha (or CXCL12) is one of the chemokines recognized by CXCR4+ cells [179].
- *β1 integrin* is a subunit of VLA4, a receptor that allows cells to cross the blood-brain barrier, particularly in the presence of inflammation as in ALS animal models and human patients. Also the β1 integrin played a critical role in NSCs migration from CSF to the parenchyma. In fact, in association with alpha 4 integrin, β1 integrin form a cell surface receptor that binds VCAM on endothelium activated by inflammatory signals and mediates the cell extracellular matrix interaction allowing the migration [180].

In addition, since β1 integrin is a NSC marker, cells positive for this marker showed a more neuralized phenotype and an increased ability to differentiate in neurons and not in other cell types.

In our work, NSCs positive for LeX, CXCR4 and β1-integrin were transplanted in the most widely used mice model of ALS, the SOD1G93A mice. This transgenic mouse, which carries multiple copies of human SOD1 gene with G93A mutation, recapitulates most of the typical hallmarks of familial and sporadic ALS patients [67]. In fact, SOD1G93A mouse model develops ALS symptoms like muscle atrophy and progressive paralysis at day 80-90, causing death at 140 days of age. Moreover, histological analyses reflect the neuropathological findings of ALS patients such as SOD1 protein aggregation, reduced number of MNs and roots, and macro- and micro-gliosis [69].

Our subset of NSCs was administered into SOD1G93A ALS mice performing intrathecal injections. Compared to systemic routes (intravenous and epidural injections), intrathecal injections allow the direct administration of cells to CSF and is far less invasive compared to local intracerebroventricular administrations. In fact, lumbar puncture is a common clinical minimal invasive procedure used for diagnostic and therapy in some neurological conditions.

In order to improve the clinical translation of this approach, the transplantation of NSCs was performed in ALS animal model after the onset of symptoms, although in an early symptomatic stages.

After intrathecal injection into the SOD1G93A transgenic mice, we analyzed whether this subpopulation of NSCs LeX+CXCR4+β1+ were able to properly engraft and migrate through the host nervous system parenchyma. We noticed that a relevant number of NSCs were engrafted in the host parenchyma. In particular, NSCs were localized in the lumbar spinal cord, near the site where the injections were performed, and also in both cervical and thoracic spinal cord, far from the site of injection. We also observed clusters of NSCs in the anterior horns area of the spinal cord, where MNs are actively degenerated. Thanks to their ability to differentiate into neuronal and glial cells, transplanted cells contributed to the host central nervous system pool constitution, although, the majority of transplanted cells maintained their undifferentiated neural stem phenotype.

Moreover, NSC transplantation was safe as revealed by absence of adverse effects like tumors, abnormal cell proliferation or neuroinflammation in treated mice. These data revealed the safety of this treatment and the ability of this NSC subset to bypass the blood brain barrier, reaching the spinal cord, and migrate through the parenchyma where in minimal part differentiate and contribute to neuronal pool.

Neuropathological analyses performed in affected mice at 120 days of age revealed that mice treated with NSCs showed an increased number of MNs and axons, higher size of nerves and preservation in NMJs. In particular, we noticed that NSC treatment determined an augmentation of NMJs fully innervated, meaning that these NMJs are functionally active. Furthermore, NSCs determined a reduction in the number of inactive or devoid NMJs. These findings revealed that NSCs exerted a therapeutic effect on ALS neuropathology by protecting MNs and their axons. In particular, this beneficial effect was at the proximal central level and also in the periphery, preserving the NMJs. In fact, at 120 days, the NSCs-treated SOD1G93A mice presented a significant improvement in NMJ innervations.

Interestingly, since the number of preserved NMJs was higher compared to the number of protecting MNs, these findings suggest that NSC transplantation in ALS mice model induced the formation and maintenance of terminal axon sprouts in the surviving MNs in order to preserve NMJs innervations. Along the disease progression in transplanted SOD1G93A, the preservation of NMJ innervations, by maintaining their integrity and inducing axonal sprouting, correlated with an improved neuromuscular phenotype, and increased survival. In fact, compared to SOD1G93A mice treated with vehicle, NSC-treated animals looked healthier, were able to walk and explore the cage and showed a relatively improving in muscle strength, as shown by a delay to incomplete the inverted hanging grid test. The improving in neuromuscular phenotype determined by transplantation of LeX+CXCR4+β1+ NSCs was associated with 16% of increased survival.

Next, we analyzed the putative mechanisms involved in neuroprotection determined by our subpopulation of NSCs. It is already established that NSCs can be therapeutic through multiple mechanisms, which include modulation of the micro-environment, reduction of inflammation, protection of MNs and neuronal circuitry and eventually cell replacement [148].

We found that LeX+CXCR4+β1+ NSCs exerted positive effects through autonomous mechanisms on MNs and non-autonomous actions that can significantly contribute to MN death in which astrocytes and microglial cells that surround MNs are involved.

Between autonomous mechanisms, our subset of NSCs determined MN protection by modulation of GSK3β pathway.

GSK3α and GSKβ are serine/threonine kinases involved in glycogen metabolism, gene transcription, apoptosis and microtubule stability [181]. In the nervous system, both isoforms of GSK3 are detected in pre and post synapses and are involved in proliferation, differentiation and neurotransmission in adult neurons [90].

In particular, GSK3β has been identified as one of the principal enzymes involved in neuronal cell death in ischemic stroke and some neurodegenerative disorders like Parkinson's disease, Alzheimer's disease and ALS [182-184].

Interestingly, in these pathological conditions, both level and activity of GSK3β are found increased. The precise mechanisms that induce the hyperactivation and overexpression of GSK3β remain to be elucidated. Since GSK3β is a constitutively active kinase, it seems that the disturbance of signaling proteins upstream of GSK3β could alter its activity and level, resulting in activation of apoptotic pathway.

It is hypothesized that excessive stresses acting on neurons could reduce the activation of PI3K, a kinase that normally phosphorylates and activates Akt. Once active, the Akt determines the phosphorylation of GSK3β in the amino-terminal serine 9 residue (Ser9), causing inhibition of its kinase activity. For this reason, some pathological conditions determine an increase in the active form of GSK3β. When hyperactivated or overexpressed, GSK3β determines a series of events involved in a variety of cellular processes. Among its substrates, CCAAT/enhancer binding protein, Myc, HSTF-1, the nuclear factor of activated T cells, β-catenin, cyclic AMP response element binding protein, tau, activator protein-1, NFkB, p53, release of cytochrome c and caspase-3 are directly affected by GSK3β [182, 184-188]. These processes result in death of neurons via activation of the mitochondrial apoptotic pathway.

Many studies showed that GSK3β expression and activity were increased in ALS MNs. *In vitro*, level of GSK3β are increased after transfection of MNs with mutant human SOD1 genes and also *in vivo*, level of this kinase is increased in the spinal cord of the ALS mice model.

Interestingly, specific inhibitors, like lithium and valproate, or using materials with GSK3β inhibitory effects reduced death of SOD1 MNs *in vitro* [189] and increased survival in ALS mouse models [175].

The putative mechanisms of protection determined by GSK3β inhibition consist in reducing death-related signals, such as cytochrome c, thereby reflecting mitochondrial injury, activated caspase-3, and cleaved forms of poly (ADP-ribose) polymerase; by restoring survival-related signals, such as HSTF-1; by decreasing inflammation-related signals such as cyclooxygenase-2 and intercellular adhesion molecule 2.

On the light of these findings, one of the possible mechanisms by which NSCs induce protection of MNs could be due to reduction of GSK3β level in spinal cord. For this reason, we analyzed level of GSK3β in the spinal cord of our cohort of mice (WT, ALS treated with vehicle or selected NSCs). Our preliminary results confirmed what observed in literature; in fact, level of GSK3β was significantly increased in the spinal cord of ALS mice compared to wild type. Despite not statistically relevant, transplantation of NSCs in affected mice seems to reduce level of GSK3β in spinal cord respect to treatment with vehicle only. Although to be confirmed, these data suggests that one of the putative mechanisms of NSC protection within MNs could be restoration of physiologically levels of GSK3β.

Among beneficial effects exerted by NSC transplantation, the reduction of macro and micro gliosis is one of the mainly effects. It is already demonstrated that in ALS, degenerating MNs release neurotoxic molecules that stimulate astrocytes and microglia to produce toxic proteins and pro-inflammatory cytokines, resulting in MN damage and initiating the vicious cycle of progressive cell death [84]. For this reason, we analyzed the effect of transplantation of LeX+CXCR4+β1+ NSCs on macro and micro glia.

Transplantation of our NSC subset in ALS mice resulted in a significant reduction of reactive astrocytosis as revealed by reduced cells positive for astrocytes markers like GFAP and s100β. Furthermore, we observed a significant reduction in microglia cells (Iba1 positive) in the spinal cord of NSC-treated SOD1G93A respect controls.

Overall, these findings suggest that NSCs transplantation has a positive effect on ALS phenotype by reverting the toxic micro-environment of murine ALS spinal cord, which can be lead to a relevant therapeutic impact.

To investigate whether NSCs exert a therapeutic effect also in a human *in vitro* model of ALS, we employed a co-culture assay using iPSC-derived MNs and ALS astrocytes derived from SOD1G93A mice model.
In this study, MNs were generated from differentiation of iPSCs obtained from skin fibroblasts of control and ALS patients (both sporadic and familial).

Promising for autologous cell therapies, the patient-specific iPSCs could be important tool to model human diseases *in vitro*, allowing a better comprehension of pathological mechanisms involved in ALS.
Both control and affected MNs derived from differentiated iPSCs undergo to degeneration when plated with astrocytes of SOD1G93A mice.
It has been demonstrated that MN death is caused by multiple toxic effects exerted by affected astrocytes. In ALS, astrocytes release toxic molecules and are no more able to produce neurotrophic factors and to up-take the glutamate causing neuro-excitotoxicity. The toxic effect of ALS astrocytes on MNs is confirmed in our co-culture system and, interestingly, MNs derived from iPSC lines of ALS patients are more vulnerable to astrocytes toxicity compared to control MNs. However, when NSCs were plated in this system, iPSC-derived MNs, WT as well as ALS, showed an improvement in terms of axonal growth and survival.

This finding reveals that LeX+CXCR4+β1 NSCs protect iPSC-MNs from toxicity triggered by murine ALS astrocytes in a non-autonomous disease *in vitro* model.

As above observed in our *in vivo* experiments performed in ALS mouse model, this subpopulation of LeX+CXCR4+β1 NSCs exerts positive effects through multiple mechanisms. Among autonomous mechanisms, the direct action of our subpopulation of NSCs on affected ALS MNs is due to the inhibition of GSK3β and production of neuroprotective factors. Our mechanistic studies of NSC beneficial effects on iPSC-derived MNs showed that NSCs in this co-culture system increased GSK3β phosphorylation, which determines inhibition of its kinase activity, a feature associated with MN survival [125].

In addition, our subset of NSCs produce neurotrophic factors such as BDNF, GDNF, NT3 and TGFα that could protect MNs from degeneration that may be neuroprotective of MNs by paracrine signaling, as our co-culture results suggest. The link between neurotrophic factors and GSK3β activation in MNs could be investigated in the future.

Finally, we showed that NSCs exerted indirect neuroprotective action contrasting the toxic astrocytes effect. In particular, NSCs block the hyper-proliferation of toxic ALS astrocytes through the activation of the TRPV1 receptor. TRPV1, transient receptor potential vanilloid-1, is a non-selective cation channel expressed on astrocytes surface.

Previous studies showed that neural and precursor stem cells (NSCs and NPCs) released endovanilloids and agonists that directly stimulate the endovanilloid receptor, TRPV1. Once activated, TRPV1 not only blocks aberrant proliferation of astrocytes and could also induces their death [190]. Indeed, treatment with TRPV1 antagonists such as iodoresiniferatoxin (I-RTX) or capsazepine (CZP) determined a reduction in protective effects exerted by NSCs.

In fact, NSCs induced astrocytes death when plated in our co-culture system; however, the addiction of TRPV1 selective inhibitors determined a reduction of NSC cytotoxicity on ALS astrocytes.

Overall, NSCs co-cultured with iPSC-derived MNs and ALS astrocytes improved MN survival and increased neurite length acting directly on MNs and indirectly contrasting toxic astrocytes effect.

6. CONCLUSIONS

Every 90 minutes someone is diagnosed with ALS, which carries a heavy clinical, economical, and social burden. Since there is no cure with a relevant effect on phenotype, ALS is associated with the urgency of an efficient, clinically meaningful therapy for this devastating and fatal disease.

Among therapeutic approaches for neurodegenerative disorders and ALS, NSC transplantation could play a relevant therapeutic role through a number of different actions, including production of neurotrophic factors, neuroprotection, and preservation of neuromuscular function. These aspects are particularly relevant for ALS treatment since many pathological mechanisms appear to contribute in its onset and progression.

Indeed, NSCs have currently attracted increasing interest as a valid and realistic treatment strategy. In particular, iPSCs represent a promising, yet unexplored, tool and their differentiation into NSCs may provide an important source of multipotent cells to transplant.

The success of a cellular approach can be achieved by increasing our understanding of stem cells features and identification of variables that play a major role in the success of transplantation.

This study contributes to developing a feasible and effective stem cell-based therapeutic strategy, testing a specific subset of NSCs in ALS animal models in order to improve their migratory capacity and efficacy.

Derived from iPSC differentiation, in our study, NSCs were selected for Lewis X, CXCR4 and β1 integrin, markers that increase the engraftment and therapeutic potential of stem cells. In fact, here we showed that intrathecal administration of LeX+CXCR4+β1 NSCs resulted in an efficient engraftment of cells, determining a protection of MNs and their axons from degeneration. This correlates with an improvement of the pathological phenotype in the ALS rodent model.

The therapeutic actions of donor LeX+CXCR4+β1 NSCs comprise amelioration of neuromuscular function, increased survival, protection of host MNs and positive changes in host-environment such as reduction of macro and micro gliosis. In particular, our findings revealed the importance of both preserving axon connections with the muscles and enhancing the capability of MN to form new NMJs via collateral sprouting to keep functional motor unit.

Moreover, no major side effects like tumors or abnormal cell proliferation were observed, confirming the safety of this approach.

One remarkable aspect of our study consists in a significant positive impact of NSC transplantation achieved in animals treated after the onset of symptoms, although in an early stages. This is an important aspect given the fact that it is likely that human trials will be performed in symptomatic phases, requiring slowing of disease progression, rather than preventing the disease.

Furthermore, we observed that this specific subpopulation of NSCs derived from human iPSCs, was able to protect affected MNs from degeneration not only in ALS mice model but also in a human *in vitro* model.

Overall, our results support the rationale and utility of deeply selecting a specific NSC population for therapeutic purpose for its impact on MN survival.

The beneficial effect of LeX+CXCR4+β1+ NSCs was achieved targeting multiple deregulated cellular and molecular mechanisms in both MNs and glial cells in ALS models.

This aspect is particularly relevant for ALS because many pathological mechanisms appear to contribute in its onset, making NSC transplantation a promising therapeutic approach for ALS.

In conclusion, this study provides evidence that the LeX+CXCR4+β1+ NSC subpopulation derived from pluripotent cells are a potentially useful therapeutic tool to ameliorate ALS disease phenotype.

Combining cell transplantation treatment with drug or gene therapy might further increase the therapeutic efficacy to a clinically significant level for ALS and other MN diseases.

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SCIENTIFIC PRODUCTIONS

Published research articles

1. Nizzardo M, **Bucchia M**, Ramirez A, Trombetta E, Bresolin N, Comi GP, Corti S. iPSC-derived LewisX+CXCR4+β1-integrin+ neural stem cells improve the amyotrophic lateral sclerosis phenotype by preserving motor neurons and muscle innervation in human and rodent models. Hum Mol Genet. 2016 Jun 6. pii: ddw163. [Epub ahead of print] PubMed PMID: 27270413.

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Abstracts

1. M. Nizzardo, C. Simone, F. Rizzo, **M. Bucchia**, S. Dametti, A. Ramirez, S. Corti. ALS therapy via transplantation of an iPSC-derived LeX+CXCR4+VLA4+ neural stem cell subpopulation. ENCALS meeting, Leuven 2014;

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Oral communications

1. June 27th, 2014: “Induced Pluripotent Stem Cells (iPSCs) as an in vitro disease model and therapeutic agent for motor neuron diseases”. MNC, Columbia University, New York;

2. October 17th, 2014: “Transferring Non autonomous cell degeneration models between EU and USA for development of effective therapies for Motor Neuron Diseases (MND): Human astrocytes generation” MNC, Columbia University, New York;

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7. July 15th, 2015: “Update in No-NMD collaborative project with Columbia University”. Ospedale Policlinico Milano;

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