CSF transplantation of a specific iPSC-derived neural stem cell subpopulation ameliorates the disease phenotype in a mouse model of spinal muscular atrophy with respiratory distress type 1

Giulia Forotti[a,1], Monica Nizzardo[a,1], Monica Bucchia[b], Agnese Ramirez[b], Elena Trombetta[c], Stefano Gatti[d], Nereo Bresolin[a,b], Giacomo Pietro Comi[a,b,2], Stefania Corti[a,b,*,2]

[a] *Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy*
[b] *Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Italy*
[c] *Flow Cytometry Service, Analysis Laboratory, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy*
[d] *Center for Surgical Research, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy*

ARTICLEINFO

ABSTRACT

Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is a genetic motor neuron disease affecting infants. This condition is caused by mutations in the *IGHMBP2* gene and currently has no cure. Stem cell transplantation is a potential therapeutic strategy for motor neuron diseases such as SMARD1, exerting beneficial effects both by replacing cells and by providing support to endogenous motor neurons. In this work, we demonstrate that human induced pluripotent stem cell (iPSC)-derived neural stem cells (NSCs) selected for the expression of specific markers, namely, Lewis X, CXCR4 and beta 1 integrin, and pretreated with neurotrophic factors and apoptosis/necroptosis inhibitors were able to effectively migrate and engraft into the host par- enchyma after administration into the cerebrospinal fluid in a SMARD1 mouse model. We were able to detect donor cells in the ventral horn of the spinal cord and observe improvements in neuropathological features, particularly preservation of the integrity of the motor unit, that were correlated with amelioration of the SMARD1 disease phenotype in terms of neuromuscular function and lifespan. This minimally invasive stem cell approach can confer major advantages in the context of cell-mediated therapy for patients with neurodegen- erative diseases.

1. Introduction

Spinal muscular atrophy with respiratory distress type I(SMARD1) is an infantile autosomal recessive genetic disease caused by mutations in the *IGHMBP2* gene, which encodes immunoglobulin-μ-binding protein 2 (IGHMBP2); the mutations reduce the level of this ubiquitous protein (Grohmann et al., 2001; Guenther et al., 2009; Jankowsky et al., 2011). SMARD1 is the second most common form of spinal muscular atrophy after spinal muscular atrophy 5q (SMA 5q). SMARD1 has a very early onset, usually within the first months of life; patients present

Abbreviations: AAV, adeno-associated virus; ALS, atrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; BTX, bungarotoxin; CSF, cerebrospinal fluid; CXCR4, C-X-C chemokine receptor type 4; DMEM/F12, Dulbecco Modified Eagles Medium/F12; EGF, epidermal growth factor; FACS, fluorescent-activated cell sorting; FGF-2, fibroblast growing factor; GDNF, glial cell-derived neurotrophic factor; GFP, green fluorescent protein; ICV, intracerebroventricular; IGF1, insulin-like growth factor 1; IGHMBP2, immunoglobulin-μ-binding protein 2; iPSCs, induced pluripotent stem cells; LeX, LewisX; MACS, magnetic-activated cell sorting; MAP 2, microtubule-associated protein 2; MNs, motor neurons; Neaa, non-essential aminoacid; NeuN, neuronal nuclei; NF-M, neurofilament-M; NGS, normal goat serum; nmd-mice, neuromuscular degeneration mice; NMJ, neuromuscular junction; NOD/SCID mice, nonobese diabetic/severe combined immunodeficiency mice; NSCs, neural stem cells; OCT4, octamer-binding transcription factor 4; Olig2, oligodendrocyte transcription actor; Pax6, Paired Box 6; SDF-1, stromal cell derived factor-1; SMA5q, spinal muscular atrophy 5q; SMARD1, spinal muscular atrophy with respiratory distress type 1; SSEA-4, stage-specific embryonic antigen-4; wt, wild-type; b- FGF, basic fibroblast growth factor

* Corresponding author at: Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Neurology Unit, IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, Via Francesco Sforza 35, 20122 Milan, Italy.
E-mail address: stefania.corti@unimi.it (S. Corti).
[1] Co-first authors.
[2] Co-senior authors.

-

symmetric, predominantly distal, muscular weakness and progressive muscular atrophy due to the degeneration of motor neurons (MNs) and severe respiratory distress caused by diaphragm palsy, with significant involvement of the autonomic nervous system in later stages of the disease (Vanoli et al., 2015). SMARD1 is a rare and fatal disease for which there is currently no available therapy; after diagnosis, patients are subjected to symptomatic treatment for some of the most important problems, such as respiratory difficulties, through prompt mechanical ventilation, and feeding with gastric tubes (Vanoli et al., 2015). Moreover, the mechanisms by which IGHMBP2 protein defects lead to MN degeneration and consequently to muscular atrophy are largely unknown, hampering the development of therapeutic strategies.

Different therapeutic approaches have been attempted at the preclinical level, from pharmacological treatment to gene therapy with adeno-associated viral vectors (Nizzardo et al., 2015; Shababi et al., 2016, 2018). Gene replacement using adeno-associated virus serotype 9 (AAV9) restores protein levels, improving neuromuscular function and ameliorating the pathological hallmarks of the disease. However, gene therapy has important limits and concerns, such as the potential negative impacts of high doses and the narrow therapeutic window, as the therapy is most effective when administered at the presymptomatic stages (Nizzardo et al., 2015; Shababi et al., 2016, 2018); thus, it is crucial to investigate new approaches, such as stem cell transplantation. Stem cell transplantation represents a potential therapy for MN dis- eases, as it not only enables protection of endogenous MNs releasing trophic factors but also provides partial replacement of damaged neural cells (Kim, 2004; Corti et al., 2008; Donnelly et al., 2012), thus ex- panding the therapeutic window to the symptomatic stages.

Preclinical rodent models of SMARD1 with neuromuscular degeneration, such as *nmd* (neuromuscular degeneration) mouse models, are commonly used, particularly B6.BKS Ighmbp2nmd-2 J mice (Cook et al., 1995; Cox et al., 1998). This murine model has a conserved *Ighmbp2* region localized on chromosome 19 instead of chromosome 11 (Cook et al., 1995) and presents a spontaneous homozygous mutation in the *Ighmbp2* gene that causes a phenotype resembling human SMARD1, with MN degeneration (Ruiz et al., 2005). The genetic defect consists of a single-nucleotide substitution (A to G) in intron 4 that causes an 80% reduction in the abundance of functional *Ighmbp2* transcripts (Maystadt et al., 2004; de Planell-Saguer et al., 2009; Fukita et al., 1993; Cox et al., 1998).

Our team and others have obtained promising results showing the beneficial effects of stem cell transplantation in MN disease models, including *nmd* mouse models (Corti et al., 2006; Simone et al., 2014; Faravelli et al., 2014), with amelioration of the neurological phenotype occurring through multiple mechanisms, particularly a paracrine mechanism (Donnelly et al., 2012). In addition, human clinical trials of stem cell transplantation treatments for other MN disorders, such as amyotrophic lateral sclerosis (ALS), have already been performed or are ongoing (Izrael et al., 2018; Abati et al., 2018); these treatments include transplantation of primary NSCs (https://www.als.net/als-research/als-clinical-trials/). Stem cells possess remarkable therapeutic potential, and iPSCs, a new resource in medicine, can be differentiated towards different cell fates, presenting the possibility of generating distinct functional cell types (Karumbayaram et al., 2009; Zhang et al., 2014). Moreover, we recently demonstrated the promising therapeutic potential of a particular subset of iPSC-derived NSCs positive for the expression of the stem cell markers Lewis X (LeX), chemokine receptor type 4 (CXCR4) and beta (β) 1 integrin, key surface proteins for cell migration and survival maintenance (Nizzardo et al., 2016). Transplantation of this subpopulation into ALS mice protects endogenous MNs and neuromuscular junctions and reduces macro- and microgliosis, bringing significant improvements in neuromuscular phenotype and survival. We have also demonstrated that transplantation of iPSC-derived NSCs is a potential therapy in *nmd* mice, considering the appropriate engraftment and differentiation of the transplanted NSCs, which were able to preserve endogenous MNs and ameliorate, if not completely rescue, the phenotype of the SMARD1 animal model (Simone et al., 2014).

Here, we show that the specific iPSC-derived NSC fraction expressing Lewis X, CXCR4 and β1 integrin, which has already been tested for ALS (Nizzardo et al., 2016), is therapeutically advantageous in terms of delivery, engraftment and therapeutic efficacy in SMARD1 when the cells are pretreated with neurotrophic factors and apoptosis/necroptosis inhibitors. Intrathecal delivery of the selected NSC fraction into SMARD1 mice resulted in significant improvements in the overall appearance, neuromuscular function and lifespan of the mice. In addition, the treatment exerted a positive effect on endogenous MN cell bodies with a strong effect at the periphery, promoting neuromuscular junction maintenance and improving muscle fiber morphology and organization. Our results confirm the potential utility of NSCs in minimally invasive transplantation for cell-mediated therapy of SMARD1.

2. Materials and methods

2.1. Cell cultures

We used iPSCs derived from a healthy subject and previously reprogrammed by us (Nizzardo et al., 2014); the cells were maintained with a specific iPSC medium (Essential 8 Medium, Life Technologies). The iPSCs were then differentiated towards an NSC line using a 10-day-long multistage protocol (Nizzardo et al., 2014): iPSC colonies were cultured with a neural differentiation medium based on DMEM/F12 (Dulbecco's modified Eagle's medium/F12, Gibco) with the addition of N2 (Gibco, Life Technologies), nonessential amino acid (NEAA) solution (Gibco) and 1 mg/ml of heparin (Sigma-Aldrich); this specific medium promoted neural differentiation.

The iPSC-derived NSCs were then selected for the expression of Lewis X (CD15), CXCR4, and β1-integrin to obtain the subpopulation of interest. Using magnetic-activated cell sorting (MACS) with magnetic beads, iPSC-derived NSCs were selected for the expression of CXCR4 (CXCR4 MicroBead Kit, Miltenyi Biotech) following the manufacturer's protocol. The purity of the MACS product was evaluated by flow cytometry; specifically, 1×10^5 cells/tube were stained at 4 °C for 10 min with 7-amino-actinomycin D (7-AAD), to exclude the dead cells from analysis, and with CXCR4-APC (mouse 1:10; Beckton Dickinson). After a few days in culture for recovery, the CXCR4+ NSC cells were then selected for CD15 expression (LeX) using MACS and the appropriate beads (CD15 MicroBead Kit, Miltenyi Biotech). The percentage of positive cells was evaluated again by flow cytometry. The CXCR4+ LeX+ cells were cultured in the neural medium previously described, then expanded and selected by fluorescence-activated cell sorting (FACS) for β1-integrin expression (CD29-PE, mouse 1:50; Beckton Dickinson) using a FACSAria Special Order Research Product (SORP) cell sorter and FACSDiva software version 8.0 (BD). Proper isotypic control antibodies were used for the negative control. All conjugated monoclonal antibodies were purchased from Becton Dickinson Biosciences (BD) and properly titrated.

After FACS cells were cultured for recovery and injected into the animal model within four days, the cells that remained in culture were tested weekly by flow cytometry to verify the maintenance of the selected markers CXCR4, CD15 and β1-integrin. For culture and cell expansion, neural medium with EGF (epidermal growth factor, Sigma-Aldrich) and FGF (fibroblast growth factor)-2 (Sigma-Aldrich) was used (Nizzardo et al., 2014).

2.2. Immunocytochemistry

iPSCs and NSCs were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X100 for 10 min and blocked with 10% normal goat serum (NGS, Jackson ImmunoResearch) and 0.25% Triton X-100 in PBS for 1 h at room temperature. The glass slides were

incubated overnight at 4 °C with specific antibodies and, after repeated washes with PBS, exposed to secondary antibodies (Alexa Fluor 488 or 594, anti-mouse, rabbit or goat, 1:1000, Life Technologies) for 1 h 30 min at room temperature. We used antibodies against the following antigens: LeX (mouse 1:10; Miltenyi), CXCR4 (rabbit 1:100; Santa Cruz), Pax6 (mouse 1:300; Biolegend), MAP2 (mouse 1:100; Sigma- Aldrich), nestin (rabbit 1:200; Millipore), NeuN (mouse 1:50, Millipore), SOX2 (mouse 1:1000; Millipore), Olig2 (mouse 1:100; Sigma-Aldrich), SSEA-4 (mouse 1:500, Chemicon), and OCT4 (mouse 1:500; Chemicon). The images were acquired with a LEICA LCS8 con- focal microscope.

2.3. Animal model

The *nmd* mouse (B6. BKS Ighmbp2nmd-2J/J, Jackson Laboratory) carries a spontaneous homozygous mutation in the murine gene *Ighmbp2* and develops a phenotype similar to the human disease. The murine model has a substitution mutation (A to G) in intron 4 that creates a cryptic splicing site interfering with the normal splicing of the transcripts. Heterozygous mice were interbred to generate homozygous mice; either *nmd* or wild-type (WT) mice were used to evaluate the treatment. The mice used in this work presented a serious phenotype with a medium to low survival time of 13.4 ± 2.8 days, in line with the original description ((Cox et al., 1998) www.jax.com).

For a primary evaluation of the cell transplantation, we used NOD/SCID mice (Jackson Laboratory) ($n = 3$), mice that are homozygous for the spontaneous mutation $Prkdc^{scid}$, known as *scid*. This murine model is characterized by the absence of functional T and B cells, lymphopenia and hypogammaglobulinemia, characteristics that make the $Prkdc^{scid}$ mouse line suitable for cell transplantation experiments (www.jax.com).

2.4. LeX + CXCR4 + β1+ stem cell transplantation

Before the transplantation of the selected subpopulation, the cells were engineered for the expression of GFP under the control of the cytomegalovirus promoter as previously described (Corti et al., 2012). The transplantation in NOD/SCID ($n = 6$) and *nmd* mice ($n = 20$) was carried out at postnatal day 1; we used a micropipette (10 μl, 30-gauge Hamilton syringe) for an intrathecal infusion of the cell suspension (2 μl with 10,000 cells/μl) in L5-L6 intervertebral space.

The selected cells were resuspended in a trophic solution rich in neurotrophic factors – brain-derived neurotrophic factor (BDNF, 10 ng/ml); glial cell-derived neurotrophic factor (GDNF, 10 ng/ml); insulin-like growth factor (IGF-1, 10 ng/ml); EGF (10 ng/ml); basic FGF (b-FGF, 20 ng/ml); and necrostatin-1 (60 μM), a selective inhibitor of necroptosis – in saline solution. All mice in treatment survived after the injection without side effects. As a control, a group of mice was treated only with vehicle using the same surgical procedure ($n = 16$) (Corti et al., 2009, 2010, 2012). Cell-treated *nmd* mice were monitored daily for an in-depth characterization of the disease hallmarks until the end stage of the disease; the researchers were blinded to the treatment ca- tegories. All mice in the experiment were treated intraperitoneally with the immunosuppressive drug FK506 1.0 mg/kg (Sigma-Aldrich) every day, starting the day before the injection. Data on survival, weight, and muscle function and ability (evaluated by the hindlimb splay) testwere monitored weekly; histological analyses of murine tissues and evaluations of the morphology of the transplanted cells were also performed ($n = 3$/group).

Animals were sacrificed when they were not able to right themselves within 30 s after being placed in a supine position (Li et al., 2000).

2.5. Histological analyses

For the histopathological analyses, NOD/SCID mice were sacrificed at 24 h and 5 months after the cell transplantation; *nmd* mice were sacrificed at postnatal day 3 and postnatal day 10. Tissues of interest, such as spinal cord, diaphragm and gastrocnemius, were isolated and preserved according to different protocols depending on the type of analysis.

Spinal cords were isolated and immersed in paraformaldehyde 4% (Sigma-Aldrich) for 4 h, then in a 10% sucrose solution in 0.12 M TPO_4 (Sigma-Aldrich) overnight; the solution was subsequently replaced with a preheated gelatin solution (Sigma-Aldrich) maintained at 37 °C for 1 h. The spinal cords were then embedded in gelatin, solidified at 4 °C, frozen in liquid isopentane, precooled at 70 °C, and stored at −80 °C. For the preservation and analysis of the muscle tissues, such as the gastrocnemius and diaphragm, muscles were directly frozen in isopentane precooled in liquid nitrogen and then maintained at −80 °C. The tissues were then mounted with Tissue-Tek Optimal Cutting Temperature compound (Sakura) and cryosectioned. The sectioned slices were 20 μm thick and were collected on SuperFrost microscope slides.

For immunohistochemistry analyses, all sections were washed with 1× PBS and blocked with 10% NGS in 1× PBS and 0.3% Triton X-100 for 1 h at room temperature. Primary antibodies were added overnight at 4 °C. For the analysis of spinal cord sections, antibodies against the following antigens were used: nestin (rabbit, 1:200, Millipore), class III β-tubulin (rabbit, 1:200; Abcam), MAP2 (mouse, 1:200; Sigma- Aldrich), anti-ChAT (goat, 1:100 Millipore), oligodendrocyte marker O4 (mouse, 1:200; Millipore), and glial fibrillary acidic protein (rabbit, GFAP, 1:200; Abcam). Anti-neurofilament M (rabbit, 1:250; Millipore) and Alexa Fluor 555-conjugated α-bungarotoxin (1:200; Life Technologies) were used to mark neuromuscular junctions (NMJs). For the analysis of GFP expression, we used an Alexa 488-conjugated anti- GFP antibody (1:1000; Molecular Probes). With unconjugated primary antibodies, we used mouse and rabbit secondary antibodies conjugated with Alexa 488 or Alexa 568 (1:1000, Life Technologies); we applied these antibodies for 1 h at room temperature.

2.6. Muscle and neuropathological analyses

The lumbar spinal cord region, gastrocnemius and diaphragm were cryosectioned for histological analyses to phenotypically characterize the hallmarks of the disease.

To evaluate the number, morphology, and size of MNs and their relative improvement after the treatment, we stained serial transverse sections of the spinal cord with NeuroTrace and observed them with a Leica Microscope (SP5). Only neurons with an area of ≥80 μm^2 and located in positions congruent with those of motor neuron groupswere counted and considered as MNs; these analyses were performed in the anterior ventral horns of the spinal cord in serial sections (Simone et al., 2014). $n = 30$ slices per group from 3 mice per group were counted for the analysis.

To evaluate the morphology, the myofiber cross-sectional size, the degeneration and the possible improvement of the muscle fibers, we stained sections of the diaphragm and gastrocnemius muscles with hematoxylin/eosin. Transverse fibers were randomly selected, and approximately 500 nonoverlapping myofibers from each muscle were captured. The cross-sectional area of each myofiber was measured to determine the mean myofiber size (Nizzardo et al., 2015).

In addition to histological staining, to verify the neuromuscular degeneration and relative recovery posttreatment, we analyzed the NMJs of the sectioned gastrocnemius muscle as previously described in the "Histological Analyses" section (Nizzardo et al., 2016). For NMJ quantification, a minimum of 100 NMJs from each muscle were randomly selected and evaluated to determine the number of denervated or degenerated junctions based on colocalization of signal from thetwo antibodies.

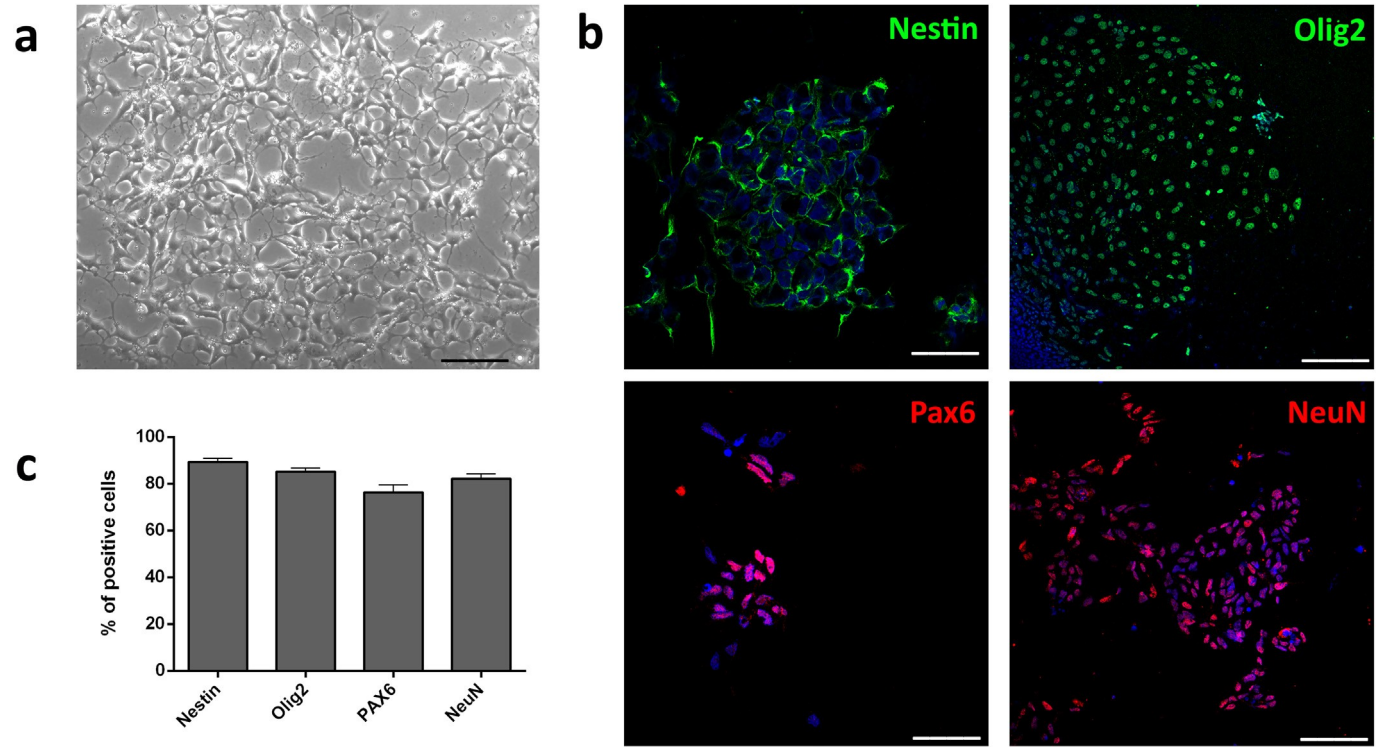

Fig. 1. iPSC differentiation into NSCs.

(a) Representative bright-field image showing NSC morphology. Scale bar: 50 μm. (b) Representative images of iPSC-derived NSCs positive for the neural precursor markers Nestin (green), Olig2 (green), Pax6 (red) and NeuN (red). The nuclei were stained with DAPI (blue signal). Scale bars: Nestin and Pax6, 50 μm; Olig2 and NeuN, 70 μm. (c) Quantification of cells positive for NSC markers. Data are shown as mean + SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Statistical analysis

For statistical analyses, GraphPad Prism 5 software was used; the null hypothesis was rejected when the p-value was < 0.05. A two-tailed unpaired Student's t-test was employed for pairwise comparisons between groups, while comparisons among more than two groups were performed with one-way analysis of variance (ANOVA). Survival was studied with Kaplan-Meier survival analysis and a log-rank test. A contingency test was used to identify any significant differences in NMJ innervation.

3. Results

3.1. iPSC differentiation in NSCs and isolation of the LeX + CXCR4 + β1 + NSC subpopulation

We used a previously established iPSC line generated by reprogramming fibroblasts from a healthy subject using a nonintegrating viral system (Nizzardo et al., 2014). The iPSCs were differentiated into NSCs using an optimized multistep protocol with neural induction medium to direct the cells towards a neuroectodermal fate (Fig. 1a). After ten days, the cells were positive for neuroepithelial markers such as Nestin (> 90%), Olig2, Pax6, and NeuN (Fig. 1b, c). The cells were self-renewing, multipotent and able to differentiate into different mature neuronal cells, changing morphologically by increasing their size and nucleus/cytoplasm ratio. The cells began to present primary extensions that led to loss of a defined colony shape.

NSCs were selected for the expression of specific markers advantageous for cell transplantation, migration and engraftment, particularly Lewis X (LeX), chemokine receptor type 4 (CXCR4), and β1-integrin (β1 or CD29) (Fig. 2a). We have already demonstrated in a murine model of another motor neuron disease that this specific subpopulation is able to efficiently integrate into the spinal cord and has beneficial effects (Nizzardo et al., 2016). Through the combined use of MACS and fluorescence-activated cell sorting (FACS), we isolated the LeX, CXCR4-, and β1-positive NSC subpopulations (Fig. 2b). These cells maintained NSC hallmarks: they were self-renewing, multipotent and able to differentiate into cells with distinct neural phenotypes (data not shown). After selection, the NSC subpopulation was expanded under adherent conditions in a particular neuronal culture medium supplemented with EGF and FGF-2. The expression of the selected markers LeX, CXCR4, and β1 was confirmed by immunofluorescence analysis (Fig. 2c).

3.2. Intrathecal transplantation of the LeX + CXCR4 + β1+ NSC subpopulation into NOD/SCID and nmd mice

To assess the migration, engraftment and fates of LeX + CXCR4 + β1+ NSCs and the success of injection after transplantation, we performed a preliminary assessment in immunodeficient NOD/SCID mice. The LeX + CXCR4 + β1+ subpopulation, genetically modified to express green fluorescent protein (GFP), was intrathecally administered into the intervertebral space of the spinal cord at L5-L6 in NOD/SCID mice at postnatal day 1, as previously described (Corti et al., 2010). Twenty-four hours after treatment, GFP+ cells were clearly visible in the cerebrospinal fluid in the area surrounding the injection site, particularly along the edge and in the ependymal canal (Fig. 3a, left panel). At 5 months after injection, the transplanted cells were still visible and even more extensively distributed in the gray matter in the anterior horns of the lumbar spinal cord, demonstrating a striking migration capacity and long-lasting engraftment (Fig. 3a, middle, right panels).

The treatment was then translated into an *nmd* SMARD1 mouse model with a severe phenotype and an average lifespan of 13.4 ± 2.8 days, consistent with the description of the original *nmd*

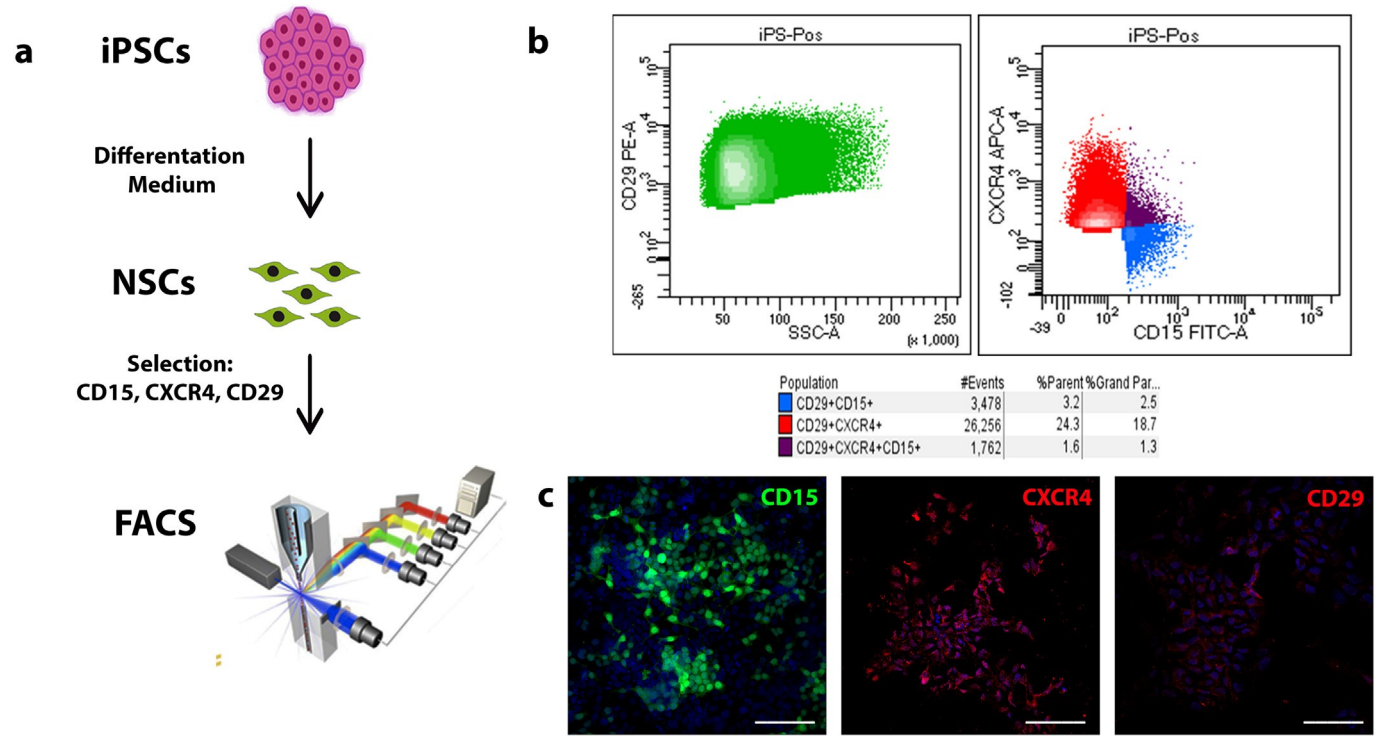

Fig. 2. Isolation of the LeX + CXCR4 + β1 + NSC subpopulation.
(a) Scheme of iPSC differentiation into NSCs and isolation of the LeX + CXCR4 + β1 + NSC subpopulation. (b) Representative flow cytometric data analysis of LeX + CXCR4 + β1 + NSCs. (c) Selected iPSC-derived NSCs expressing CD15 (green), CXCR4 (red), and CD29 (β1 integrin, red). The nuclei were stained with DAPI (blue signal). Scale bars: CD15 and CD29, 50 μm; CXCR4, 70 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strain ((Cox et al., 1998), www.jax.org). We administered 2×10^4 cells into the cerebrospinal fluid by intrathecal administration in *nmd* mice as described for NOD/SCID mice at postnatal day 1, and we detected GFP+ cells in the ventral horns of the spinal cord, the site of major motor neuronal degeneration (Fig. 3b). GFP+ cells, after the injection in L5-L6 intervertebral space, were detected through the entire lumbar spinal cord. Interestingly, a consistent proportion of GFP+-engrafted cells continued to express NSC markers, such as Nestin (70.7 ± 5.5%) (Fig. 3b, right panel, Fig. 3c), suggesting the maintenance of a neural stem cell/precursor phenotype even after engraftment. Only a small percentage of NSCs differentiated into neurons 5.89 ± 1.67% (TuJ1-GFP+ cells) or astrocytes 4.65 ± 1.89% (GFAP-GFP+ cells) (Fig. 3c). The oligodendrocyte marker O4 was negative. We did not detect the expression of the MN marker ChAT in donor cells, suggesting that in this experimental setting, the grafted cells did not become mature MNs. These data suggest that rather than substituting the endogenous MN cell population, donor NSCs exert a paracrine action on them.

To improve cell survival during transplantation, we used cell suspensions with a series of neurotrophic factors, such as BDNF, b-FGF, GDNF, IGF1, EGF and Necrostatin-1. All mice in the experiments were intraperitoneally injection with an immunosuppressive drug, FK506, once per day at a dose of 1.0 mg/kg. After cell treatment, the mice did not present major side effects, such as abnormal cell proliferation or inflammatory responses.

3.3. *Improvement of the neuromuscular phenotype and increase in nmd mouse survival after LeX + CXCR4 + β1+ NSC transplantation*

To evaluate the effects of LeX + CXCR4 + β1+ NSC transplantation on the SMARD1 phenotype, we injected an expanded cohort of mice ($n = 20$) according the same protocol, and as controls, we used *nmd* mice injected only with vehicle ($n = 16$). The injected *nmd* mice were monitored daily with regard to the phenotypic disease hallmarks, weight and survival until the end stage. Vehicle-treated *nmd* mice presented the first clinical symptoms at the early stage by the end of the first postnatal week, showing muscle weakness starting from the hind limbs, notable dorsal contraction and impaired locomotor activity. Moreover, when suspended by the tail, vehicle-treated *nmd* mice were not able to extend their hindlimbs, clench them, or grab the cage (Fig. 4a, middle panel). The reduced hindlimb extension caused im- proper motor activity, severely hampering correct movement on all four paws. As the disease progressed, the muscle weakness worsened and involved the forelimbs; vehicle-treated *nmd* mice presented reduced survival, with cases of paralysis and death in the first postnatal week and a maximum lifespan of two weeks. Intrathecal transplantation with LeX + CXCR4 + β1+ NSCs significantly improved the muscular condition of the treated *nmd* mice compared to their vehicle-treated littermates. Mice that received the transplants maintained autonomous movement; if suspended by the tail, they were able to stretch their hindlimbs (Fig. 4a, right panel). The treated mice also explored the cage with interest, supporting the positive effects of the cell treatment on muscular performance.

Another important result was the difference in body weight between the cell-treated *nmd* mice and their vehicle-treated littermates. At birth, no particular discrepancy was observed between the two groups; however, differences became clearly visible after the first days of life. LeX + CXCR4 + β1+ NSC treatment significantly increased body weight as recorded at 7 and 14 days of life, showing another beneficial effect of cell transplantation as a therapeutic approach ($p < .001$ and $p < .01$, respectively, Fig. 4b). Importantly, the treated *nmd* mice also had significantly longer lifespans than the vehicle-treated ones; for transplanted mice, death occurred after a median of 22.7 ± 5.8 days, while for vehicle mice, death occurred after a median of 13.4 ± 2.8 days (Kaplan-Meier survival curves, $p < .0001$, $\chi^2 = 26.64$, Fig. 4c).

Our results demonstrated an overall positive impact, showing that

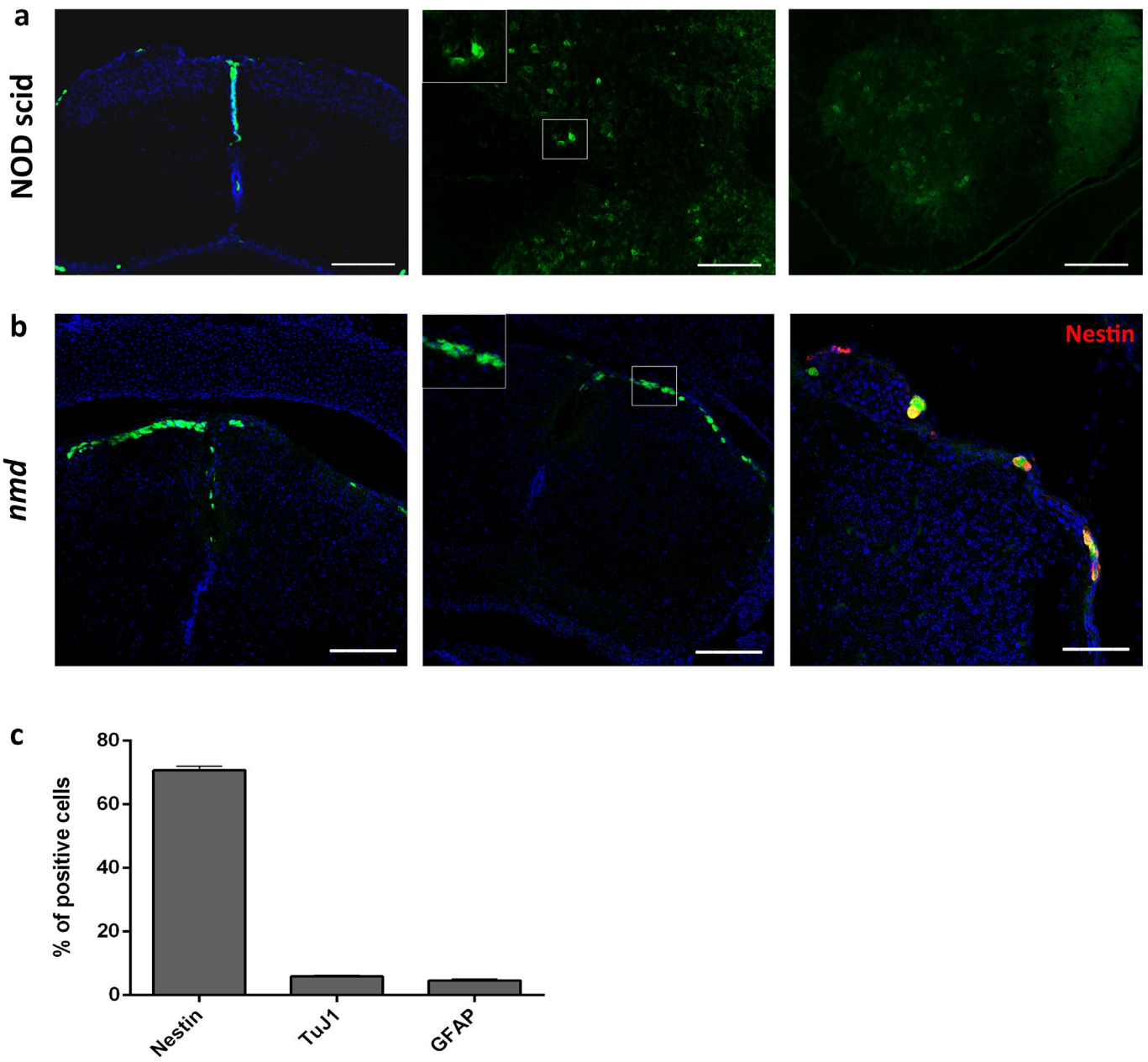

Fig. 3. Intrathecal transplantation and engraftment of the LeX + CXCR4 + β1+ NSC subpopulation in NOD-scid and *nmd* mice.
(a) Representative images of analyzed mice ($n = 3$) in the NOD-scid mouse model. Twenty-four hours after injection, transplanted GFP+ cells were clearly visible in the cerebrospinal fluid (left panel), and after 5 months, they were detected in the gray matter in the anterior horns of the lumbar spinal cord (middle, right panels). In the insect higher magnification of the area marked in the middle panel. The nuclei were stained with DAPI in the left panel (blue signal). Scale bars: 24 h, 125 μm; 5 months, 75 μm. (b) Representative images of analyzed mice ($n = 3$) in the *nmd* mouse model. Forty-eight hours after injection, GFP+ cells had engrafted into the spinal cord, were detectable in the cerebrospinal fluid and in the ventral horns of the spinal cord (left, middle panel), and had maintained positivity for the NSC marker Nestin (right panel, red). In the insect higher magnification of the area marked in the middle panel. The nuclei were stained with DAPI (blue signal). Scale bar: 50 μm, 30 μm (b, right panel). (c) Quantification of the acquired phenotype by the engrafted cells: a consistent proportion of GFP + -engrafted cells continued to express NSC markers, such as Nestin, while a minimal percentage of NSCs differentiated in neurons (TuJ1-GFP+ cells), and astrocytes (GFAP-GFP + cells). Data are presented as mean + SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LeX + CXCR4 + β1+ NSC transplantation improved the pathological phenotype of treated mice in terms of survival, growth and muscular function.

3.4. *Protective effect of LeX + CXCR4+ β1+ NSC transplantation on MNs*

SMARD1 is characterized by significant and progressive neuromuscular degeneration; to verify the effects of transplantation on neuropathological hallmarks, we analyzed murine tissues derived from cell-treated *nmd* mice and vehicle-treated *nmd* mice on postnatal day 10. The disease is characterized by progressive MN degeneration. Histological analysis of spinal cord lumbar sections L1-L5 revealed marked loss and severe degeneration of MNs in vehicle-treated *nmd* mice compared to their wild-type (WT) littermates. Transplantation increased the number and size of endogenous MNs (Fig. 5a, right panel). LeX + CXCR4 + β1+ NSC transplantation promoted MN protection, leading to significantly less loss ($p < .0001$, Fig. 5b) and greater size ($p < .0001$, Fig. 5c) than observed in vehicle-treated mice.

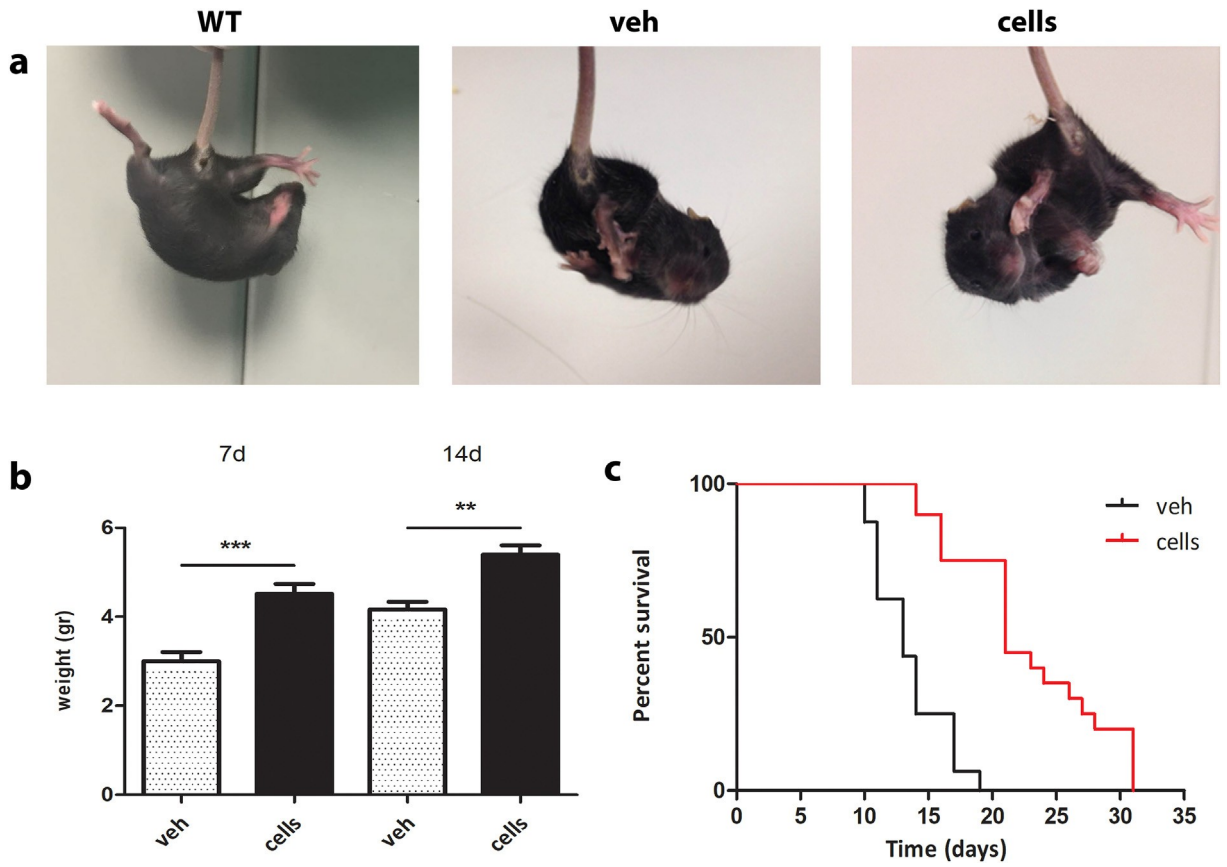

Fig. 4. Improvements in the neuromuscular phenotype and increases in *nmd* mouse survival after Lex + CXCR4 + β1+ NSC transplantation.
(a) Representative images showing the ability of cell-transplanted *nmd* mice to stretch their hindlimbs when suspended by the tail (right panel); vehicle-treated *nmd* mice (middle panel) were not able to do so. (b) The mean body weight of cell-treated *nmd* mice was significantly higher than that of vehicle-treated *nmd* mice at two different time points, postnatal day 7 (7 d, ***$p < .001$) and postnatal day 14 (14 d, **$p < .01$) (Student's *t*-test). The values represent the means ± SEM. (c) Kaplan-Meier survival curves of cell-treated *nmd* mice and vehicle-treated *nmd* mice demonstrating that cell transplantation significantly extended the median survival by 8 days ($p < .0001$, $\chi^2 = 26.64$, Kaplan–Meier log rank test). Cell-treated *nmd* mice, $n = 20$; vehicle-treated *nmd* mice, $n = 16$. WT: wild-type mice; veh: vehicle-treated *nmd* mice; cells: cell-treated *nmd* mice.

3.5. *Protective effect of LeX + CXCR4+ β1 NSC transplantation on NMJs and muscle tissues*

Nmd mice presented degeneration of NMJs, whose denervation increased with progression of the disease; MN endplates were immature, with incompletely developed subsynaptic clefts and folds or even an absence of subsynaptic invagination and button-shaped structures (Villalon et al., 2018). This pathological hallmark was observed in nearly 70% of neuromuscular plates from the gastrocnemius muscles of vehicle-treated *nmd* mice at day postnatal 10 (Fig. 6a middle panel). In contrast, LeX + CXCR4 + β1+ NSC transplantation significantly counteracted NMJ degeneration with recovery of peripheral synapses; nearly 60% of endplates presented normal structures, and the junctions were innervated with correct "pretzel-like" structures, which indicates a functional network of acetylcholine receptors (Fig. 6a, b, $p < .0001$, right).

Vehicle-treated *nmd* mice presented consistent myopathy linked to denervation, with wide variability in fiber size, lipid infiltration and necrotic myofibers in the studied muscles, as previously described (Grohmann et al., 2004). LeX + CXCR4 + β1+ NSC treatment exerted beneficial effects on fiber morphology in the gastrocnemius and diaphragm muscles, improving muscle fiber shape, increasing myofiber caliber (internal diameter) and reducing lipid and connective inclusions (Fig. 7a, b). The increase in myofiber caliber in treated mice was significant in both the gastrocnemius and the diaphragm ($p < .0001$ Fig. 7c, d).

The data demonstrated the power of NSC transplantation to ameliorate peripheral neuropathology, particularly with regard to NMJ innervation maintenance and muscle organization.

4. Discussion

SMARD1 is a rare, infantile-onset genetic disease that is fatal and has no cure. SMARD1 is characterized by motor neuron degeneration with muscular atrophy. The aim of this study was to therapeutically modify these pathological hallmarks through stem cell transplantation using a selected NSC population derived from iPSCs. iPSCs can be easily obtained by reprogramming somatic cells; these stem cells are able to self-renew, allowing them to produce an immense number of cells that can be used as in vitro disease cell models and as sources for transplantation without ethical concerns. However, NSCs are preferred to iPSCs for neurodegenerative therapies, as the former do not originate teratomas, can be easily obtained through reproducible protocols and are already directed towards neural fates. To increase the therapeutic benefit in our study, we selected iPSC-derived NSCs for the expression of specific superficial proteins that confer enhanced migration ability and amplify sensitivity to recruitment to the area of interest. Our team has already demonstrated that isolation of this specific subpopulation improves the overall therapeutic effect in ALS mice after intrathecal or intravenous injection (Nizzardo et al., 2014, Nizzardo et al., 2016). Selection of the optimal cell donor population is crucial for obtaining the greatest therapeutic benefit. We isolated NSCs positive for LeX, CXCR4 and β1-integrin. LeX is a marker characteristic of NSCs; CXCR4 is a specific receptor for a chemokine present in the cerebrospinal fluid,

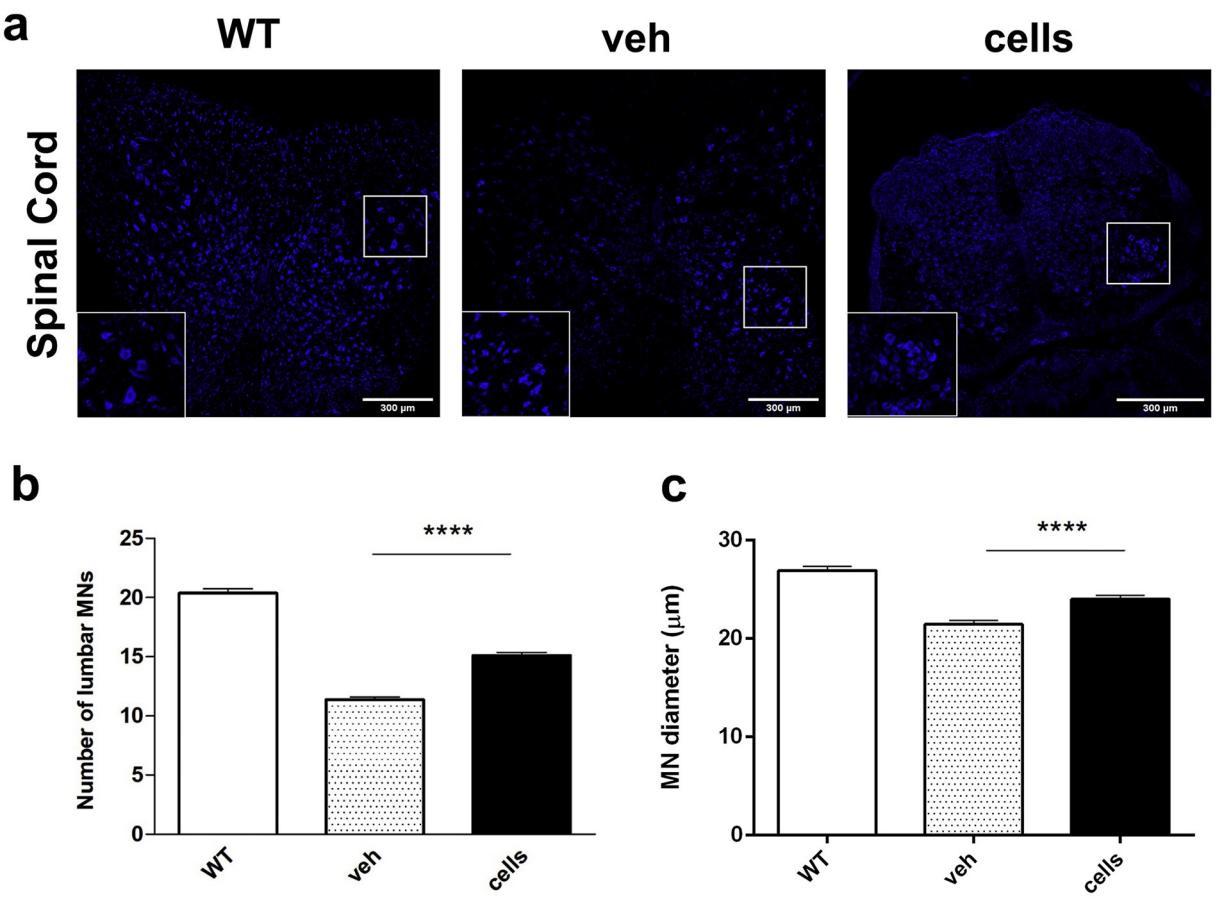

Fig. 5. Protective effect of LeX + CXCR4 + B1+ NSC transplantation on MNs.
Representative images of NeuroTrace Nissl (blue)-stained lumbar spinal cord MNs of wild-type mice, vehicle-treated *nmd* mice and cell-treated *nmd* mice at postnatal day 10. LeX + CXCR4 + β1+ NSC transplantation promoted MN protection by improving endogenous MN number and size. Scale bar: 125 µm. (b) Quantification of MNs in the lumbar spinal cord. MN number and dimension (c) were significantly greater in the cell-treated group than in the vehicle group (****$p < .0001$, mean ± SEM, $n = 30$ slices counted/group, 3 mice/group; one-way ANOVA). WT: wild-type mice; veh: vehicle-treated *nmd* mice; cells: cell-treated *nmd* mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stromal cell-derived factor 1 (SDF-1), whose action promotes recruitment to the central nervous system and transendothelial migration (Corti et al., 2007); and β1-integrin is a subunit that, in combination with an α chain, forms a heterodimeric integrin that plays an important role in migration and adhesion (Nizzardo et al., 2013) Moreover, the NSCs were resuspended in a trophic solution rich in neurotrophic factors important to improve the survival of the transplanted cells and enable the maintenance of the neural phenotype (Oliveira et al., 2013); trophic molecules are also used to maintain neural functions in neurodegenerative diseases (Esfahani et al., 2015). We chose factors such as BDNF, a neurotrophin essential in the nervous system; GDNF, which is important for neural development; IGF-1, which is crucial for motor neuron protection; FGF, which is used as a growth factor; EGF, which plays a role in the development of the mammalian nervous system; and necrostatin-1, a selective inhibitor of necroptosis that inhibits the degeneration of neural cells after exposure to toxic stimuli (Qinli et al., 2013).

Our NSC subpopulation was directly injected into the cerebrospinal fluid in the L5-L6 intervertebral space to facilitate NSC diffusion in the lumbar spinal region of the treated mice. Intrathecal transplantation of the selected cell suspension had the advantage of delivering the cells into a region close to a major MN degeneration area in SMARD1 (the lumbar area). Since the most affected section of the spinal cord in SMARD1 is the lumbar portion, it is very important that our subpopulation can migrate and spread throughout this section and not be restricted to the injection area (L5-L6). Our previous studies have demonstrated how minimally invasive administration techniques, such as intrathecal injection, effectively permit cell migration into the central nervous system (Corti et al., 2008, 2009, 2010) to obtain the desired therapeutic effect. The engraftment and migration ability of the cells and their ability to reach the spinal cord were first demonstrated in immunosuppressed mice (NOD/SCID) and confirmed in the SMARD1 model mice, *nmd* mice, in which transplantation led to substantial amelioration of the disease phenotype, with reduced muscular and MN degeneration and partial recovery in NMJs. Indeed, MNs seemed to be preserved, with increased soma size. In *nmd* mice, NMJ denervation is substantial in distal muscles compared to proximal muscles (Villalon et al., 2018); cell administration recovered the presynaptic and postsynaptic architecture of the plates with morphologies comparable to those in WT mice. Even muscle tissue morphology and organization were positively influenced by transplantation, with increased fiber caliber and improved definition in both the diaphragm and the gastrocnemius.

The preservation of endogenous NMJs and the improvements in muscle tropism and organization were more pronounced than the increases in MN soma numbers, suggesting an important role of the transplanted cells in preserving the connections between MN axons and muscles and enhancing the ability of MNs to form new NMJs via collateral sprouting to maintain the functional motor unit, as already demonstrated in our previous work with the same NSC population (Nizzardo et al., 2016).

In SMARD1 – as in other motor neuron diseases, such as ALS –

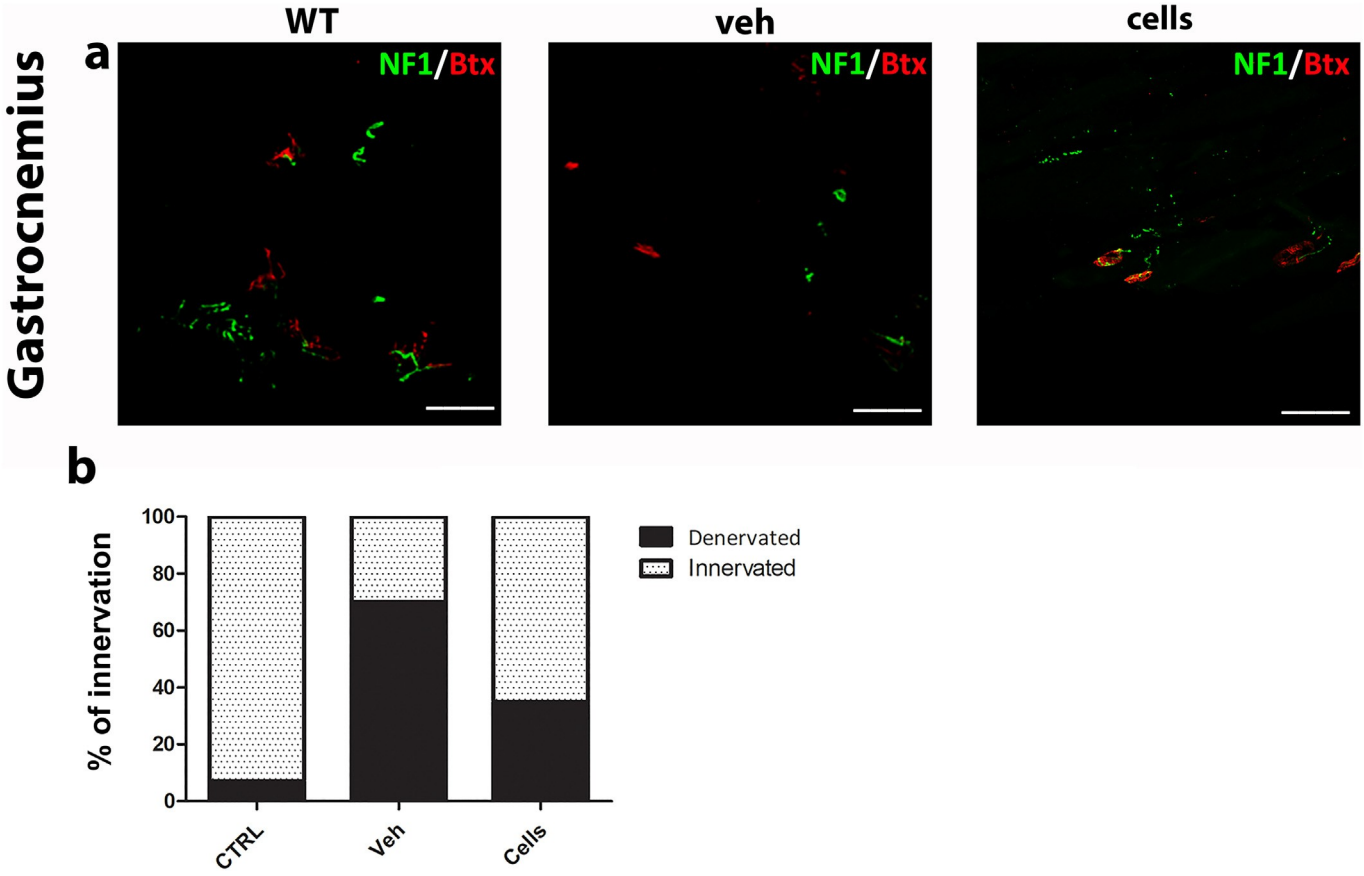

Fig. 6. Protective effect of LeX + CXCR4 + B1+ NSC transplantation on NMJs.
Representative images of NMJs labeled with Neurofilament-M (NF-M, green) and Bungarotoxin (BTX, red) in gastrocnemius muscle tissue at postnatal day 10. LeX + CXCR4 + β1+ NSC transplantation enabled the preservation of NMJs. Scale bar: 50 μm. (b) Quantification of innervated NMJs. LeX + CXCR4 + β1+ NSC transplantation reduced NMJ degeneration with recovery of peripheral synapses ($p < .0001$, contingency test, right). WT: wild-type mice; veh: vehicle-treated *nmd* mice; cells: cell-treated *nmd* mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

progressive motor neuron loss associated with the retraction of axons from NMJs is not sufficiently compensated by the axonal collateral sprouts from spare MNs, causing progressive irreversible muscle paralysis. Our present study demonstrated that a specific subpopulation of human-derived NSCs could protect SMARD1 MNs from degeneration, enhancing their ability to maintain NMJs and form novel collateral sprouting. The modest degree of muscle denervation in proportion to a more pronounced extent of motor neuron cell death suggested that muscles in NSC-transplanted *nmd* mice were reinnervated by terminal and collateral sprouts extending from spare MNs that were in a pro-regenerative state after NSC administration. Overall, while eliciting a positive action on the motor neuron soma, our NSC subpopulation also exerted a positive action at the periphery, promoting NMJ maintenance and collateral axonal sprouting. Mechanisms that protect only one cell compartment, either the motor neuronal cell body or the axonal process, do not appear adequate to preserve the integrity of the whole motor unit, and protection of both the soma and the peripheral side of the MN are necessary to have a significant impact on MN disease (Nizzardo et al., 2016). Thus, our study further supports the relevance of both protecting the connection of the axon with the muscles and promoting the capacity of MNs to form new NMJs via collateral sprouting to preserve the functional motor unit.

As we have already discussed, this NSC therapy generated multiple improvements and positive effects; however, the treated mice continued to present partial motor neuron loss with some remaining denervation of NMJs and partial loss of muscle tissue definition. Our data suggest that cell transplantation likely delayed the onset of the disease and slowed its progression, ultimately delaying death. We believe that the tested approach can be optimized, for instance, through the use of repeated injections or improved cocktails of neurotrophic factors. Indeed, we and other groups have already observed that many of the beneficial effects of transplantation are related mainly to the action of the trophic factors released (Oliveira et al., 2013; Nizzardo et al., 2016) in exerting protective effects against endogenous cell degeneration and ameliorating toxic environments (Faravelli et al., 2014).

Since SMARD1 is a monogenic disorder, gene therapy appears to be the most suitable approach. The Corti and Lorson groups used AAV9 to deliver a WT copy of the *IGHMBP2* gene into *nmd* mice through systemic or intracerebroventricular (ICV) injection and found a striking rescue of neuromuscular functions and survival as a result (Nizzardo et al., 2015; Shababi et al., 2016). However, such treatment has a very narrow therapeutic window, and gene substitution is completely efficacious only if it starts at birth in presymptomatic mice; at this stage, affected children usually have not yet been diagnosed. For this reason, there is an urgent need to find a strategy to improve gene therapy efficacy at the symptomatic stages, and NSC transplantation could be a suitable approach. Combined NSC transplantation and gene therapy could restore defective genes, correct etiopathological mechanisms, and improve symptomatic phenotypes. Such combined treatment could have important implications for SMARD1 and other monogenic neurodegenerative diseases.

The promising results obtained from this study demonstrate the possibility of using cell transplantation, either alone or in combination with gene therapy, to modify the course of SMARD1. The clinical translation of cell-based strategies for MN disorders is becoming possible given the promising results obtained in ALS (www.clinicaltrial.

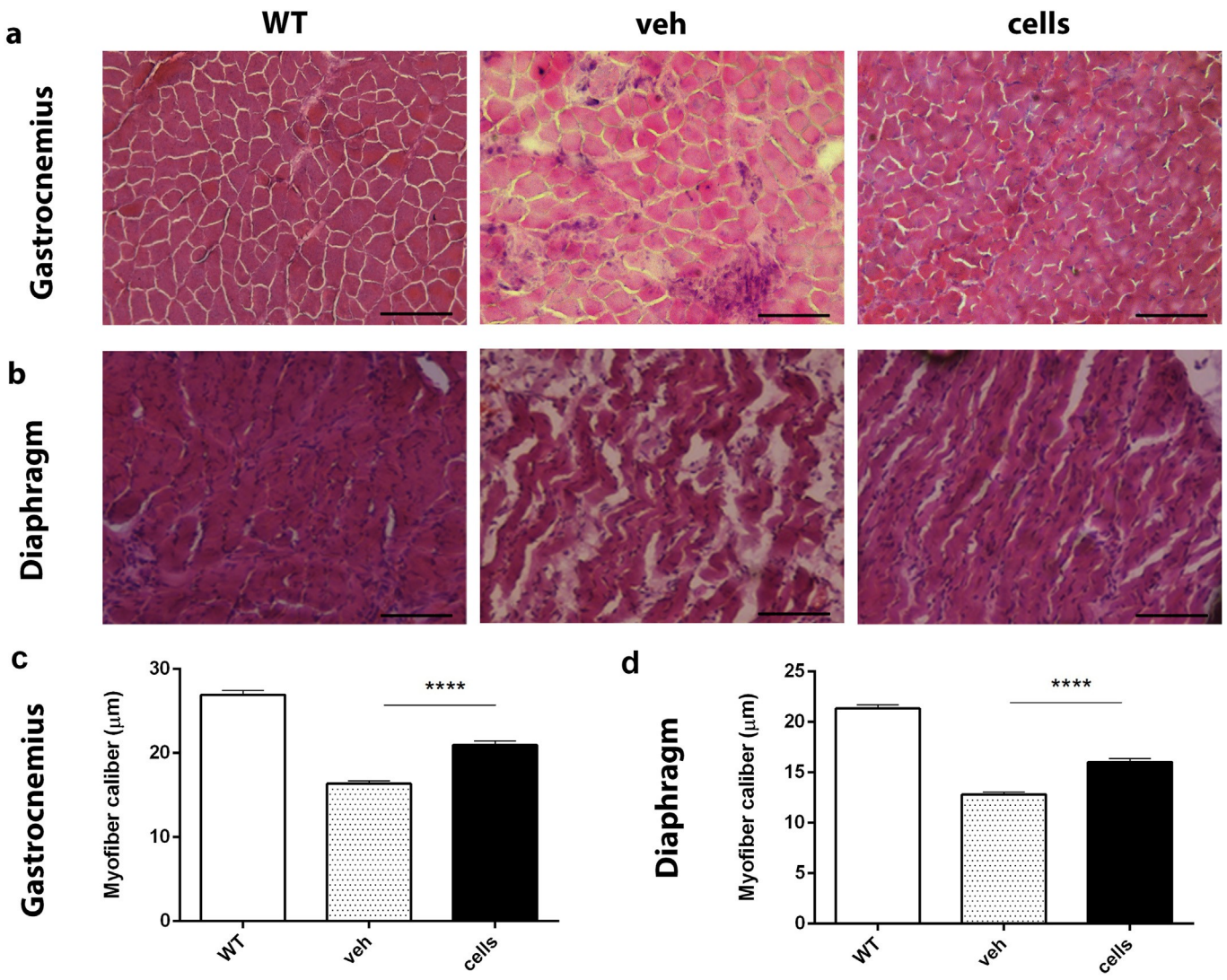

Fig. 7. Protective effect of LeX + CXCR4 + B1+ NSC transplantation on muscle tissues.
(a, b) Representative images of hematoxylin and eosin (H&E) staining in gastrocnemius (a) and diaphragm (b) muscles of WT mice, vehicle-treated *nmd* mice and cell-treated *nmd* mice. LeX+CXCR4+β1+NSC treatment improved muscle fibers by increasing myofiber caliber and reducing lipid and connective inclusions. Scale bar: 100 μm. (c,d) Myofiber caliber was significantly greater in the cell-treated group than in the vehicle group (****$p < .0001$, mean ± SEM, $n = 30$ slices counted/group, 3 mice/group; one-way ANOVA). WT: wild-type mice; veh: vehicle-treated *nmd* mice; cells: cell-treated *nmd* mice.

gov), demonstrating the safety and clinical feasibility of these therapeutic strategies.

Funding

This study was supported by the AFM Telethon (to GPC, N°#17489), Finalizzata 2016 (to GPC, RF-2016-02362317), Cariplo Foundation (to MN, 2015-0776). This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 778003 to SC; Animal experiments partially funded by Italian fiscal contribution "5 × 1000" 2014 - MIUR - devolved to Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico.

Acknowledgements

The authors wish to thank Associazione Amici del Centro Dino Ferrari for support.

Appendix A. Supplementary data

References

Abati, E., Bresolin, N., Comi, G.P., Corti, S., 2018. Preconditioning and cellular engineering to increase the survival of transplanted neural stem cells for motor neuron disease therapy. Mol. Neurobiol. 56 (5), 3356–3367.
Cook, S.A., Johnson, K.R., Bronson, R.T., Davisson, M.T., 1995. Neuromuscular degeneration (nmd): a mutation on mouse chromosome 19 that causes motor neuron degeneration. Mamm. Genome: Off. J. Int. Mammalian Genome Soc. 6 (3), 187–191.
Corti, S., Locatelli, F., Papadimitriou, D., Donadoni, C., Del Bo, R., Crimi, M., et al., 2006. Transplanted ALDHhiSSClo neural stem cells generate motor neurons and delay disease progression of nmd mice, an animal model of SMARD1. Hum. Mol. Genet. 15 (2), 167–187.
Corti, S., Locatelli, F., Papadimitriou, D., Del Bo, R., Nizzardo, M., Nardini, M., et al., 2007. Neural stem cells LewisX+ CXCR4+ modify disease progression in an amyotrophic lateral sclerosis model. Brain J. Neurol. 130, 1289–1305 Pt 5.
Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., et al., 2008. Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. J. Clin. Invest. 118 (10), 3316–3330.
Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Del Bo, R., et al., 2009.

Motoneuron transplantation rescues the phenotype of SMARD1 (spinal muscular atrophy with respiratory distress type 1). J. Neurosci. 29 (38), 11761–11771.
Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., et al., 2010. Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice. Brain J. Neurol. 133, 465–481 Pt 2.
Corti, S., Nizzardo, M., Simone, C., Falcone, M., Nardini, M., Ronchi, D., et al., 2012. Genetic correction of human induced pluripotent stem cells from patients with spinal muscular atrophy. Sci. Transl. Med. 4 (165), 165ra2.
Cox, G.A., Mahaffey, C.L., Frankel, W.N., 1998. Identification of the mouse neuromuscular degeneration gene and mapping of a second site suppressor allele. Neuron 21 (6), 1327–1337.
de Planell-Saguer, M., Schroeder, D.G., Rodicio, M.C., Cox, G.A., Mourelatos, Z., 2009. Biochemical and genetic evidence for a role of IGHMBP2 in the translational machinery. Hum. Mol. Genet. 18 (12), 2115–2126.
Donnelly, E.M., Lamanna, J., Boulis, N.M., 2012. Stem cell therapy for the spinal cord. Stem Cell Res Ther 3 (4), 24.
Esfahani, M., Karimi, F., Afshar, S., Niknazar, S., Sohrabi, S., Najafi, R., 2015. Prolyl hydroxylase inhibitors act as agents to enhance the efficiency of cell therapy. Expert. Opin. Biol. Ther. 15 (12), 1739–1755.
Faravelli, I., Riboldi, G., Nizzardo, M., Simone, C., Zanetta, C., Bresolin, N., et al., 2014. Stem cell transplantation for amyotrophic lateral sclerosis: therapeutic potential and perspectives on clinical translation. Cell. and Mol. Life Sci.: CMLS 71 (17), 3257–3268.
Fukita, Y., Mizuta, T.R., Shirozu, M., Ozawa, K., Shimizu, A., Honjo, T., 1993. The human S mu bp-2, a DNA-binding protein specific to the single-stranded guanine-rich sequence related to the immunoglobulin mu chain switch region. J. Biol. Chem. 268 (23), 17463–17470.
Grohmann, K., Schuelke, M., Diers, A., Hoffmann, K., Lucke, B., Adams, C., et al., 2001. Mutations in the gene encoding immunoglobulin mu-binding protein 2 cause spinal muscular atrophy with respiratory distress type 1. Nat. Genet. 29 (1), 75–77.
Grohmann, K., Rossoll, W., Kobsar, I., Holtmann, B., Jablonka, S., Wessig, C., et al., 2004. Characterization of Ighmbp2 in motor neurons and implications for the pathomechanism in a mouse model of human spinal muscular atrophy with respiratory distress type 1 (SMARD1). Hum. Mol. Genet. 13 (18), 2031–2042.
Guenther, U.P., Handoko, L., Varon, R., Stephani, U., Tsao, C.Y., Mendell, J.R., et al., 2009. Clinical variability in distal spinal muscular atrophy type 1 (DSMA1): determination of steady-state IGHMBP2 protein levels in five patients with infantile and juvenile disease. J. Mol. Med. 87 (1), 31–41.
Izrael, M., Slutsky, S.G., Admoni, T., Cohen, L., Granit, A., Hasson, A., et al., 2018. Safety and efficacy of human embryonic stem cell-derived astrocytes following intrathecal transplantation in SOD1(G93A) and NSG animal models. Stem Cell Res Ther 9 (1), 152.
Jankowsky, A., Guenther, U.P., Jankowsky, E., 2011. The RNA helicase database. Nucleic Acids Res. 39 (Database), D338–D341.
Karumbayaram, S., Novitch, B.G., Patterson, M., Umbach, J.A., Richter, L., Lindgren, A., et al., 2009. Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. Stem Cells 27 (4), 806–811.
Kim, S.U., 2004. Human neural stem cells genetically modified for brain repair in neurological disorders. Neuropathol.: off. J. Japan. Soc. Neuropathol. 24 (3), 159–171.
Li, Y., Chopp, M., Chen, J., Wang, L., Gautam, S.C., Xu, Y.X., Zhang, Z., 2000. Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. J. Cereb. Blood Flow Metab. 20, 1311–1319.
Maystadt, I., Zarhrate, M., Landrieu, P., Boespflug-Tanguy, O., Sukno, S., Collignon, P., et al., 2004. Allelic heterogeneity of SMARD1 at the IGHMBP2 locus. Hum. Mutat. 23 (5), 525–526.
Nizzardo, M., Simone, C., Falcone, M., Riboldi, G., Comi, G.P., Bresolin, N., et al., 2013. Direct reprogramming of adult somatic cells into other lineages: past evidence and future perspectives. Cell Transplant. 22 (6), 921–944.
Nizzardo, M., Simone, C., Rizzo, F., Ruggieri, M., Salani, S., Riboldi, G., et al., 2014. Minimally invasive transplantation of iPSC-derived ALDHhiSSCloVLA4+ neural stem cells effectively improves the phenotype of an amyotrophic lateral sclerosis model. Hum. Mol. Genet. 23 (2), 342–354.
Nizzardo, M., Simone, C., Rizzo, F., Salani, S., Dametti, S., Rinchetti, P., et al., 2015. Gene therapy rescues disease phenotype in a spinal muscular atrophy with respiratory distress type 1 (SMARD1) mouse model. Sci. Adv. 1 (2), e1500078.
Nizzardo, M., Bucchia, M., Ramirez, A., Trombetta, E., Bresolin, N., Comi, G.P., Corti, S., 2016 Aug 1. 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. 25 (15), 3152–3163.
Oliveira, S.L., Pillat, M.M., Cheffer, A., Lameu, C., Schwindt, T.T., Ulrich, H., 2013. Functions of neurotrophins and growth factors in neurogenesis and brain repair. Cytometry Part A: J. Int. Soc. Anal. Cytol. 83 (1), 76–89.
Qinli, Z., Meiqing, L., Xia, J., Li, X., Weili, G., Xiuliang, J., et al., 2013. Necrostatin-1 inhibits the degeneration of neural cells induced by aluminum exposure. Restor. Neurol. Neurosci. 31 (5), 543–555.
Ruiz, R., Lin, J., Forgie, A., Foletti, D., Shelton, D., Rosenthal, A., et al., 2005. Treatment with trkC agonist antibodies delays disease progression in neuromuscular degeneration (nmd) mice. Hum. Mol. Genet. 14 (13), 1825–1837.
Shababi, M., Feng, Z., Villalon, E., Sibigtroth, C.M., Osman, E.Y., Miller, M.R., et al., 2016. Rescue of a mouse model of spinal muscular atrophy with respiratory distress type 1 by AAV9-IGHMBP2 is dose dependent. Mol. Ther.: J. Am. Soc. Gene Ther. 24 (5), 855–866.
Shababi, M., Villalon, E., Kaifer, K.A., DeMarco, V., Lorson, C.L., 2018. A direct comparison of IV and ICV delivery methods for gene replacement therapy in a mouse model of SMARD1. Mol. Ther. Methods & Clin. Dev. 10, 348–360.
Simone, C., Nizzardo, M., Rizzo, F., Ruggieri, M., Riboldi, G., Salani, S., et al., 2014. iPSC-derived neural stem cells act via kinase inhibition to exert neuroprotective effects in spinal muscular atrophy with respiratory distress type 1. Stem cell Rep. 3 (2), 297–311.
Vanoli, F., Rinchetti, P., Porro, F., Parente, V., Corti, S., 2015. Clinical and molecular features and therapeutic perspectives of spinal muscular atrophy with respiratory distress type 1. J. Cell. Mol. Med. 19 (9), 2058–2066.
Villalon, E., Shababi, M., Kline, R., Lorson, Z.C., Florea, K.M., Lorson, C.L., 2018. Selective vulnerability in neuronal populations in nmd/SMARD1 mice. Hum. Mol. Genet. 27 (4), 679–690.
Zhang, J., Ho, J.C., Chan, Y.C., Lian, Q., Siu, C.W., Tse, H.F., 2014. Overexpression of myocardin induces partial transdifferentiation of human-induced pluripotent stem cell-derived mesenchymal stem cells into cardiomyocytes. Phys. Rep. 2 (2), e00237.