Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice

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Spinal muscular atrophy, characterized by selective loss of lower motor neurons, is an incurable genetic neurological disease leading to infant mortality. We previously showed that primary neural stem cells derived from spinal cord can ameliorate the spinal muscular atrophy phenotype in mice, but this primary source has limited translational value. Here, we illustrate that pluripotent stem cells from embryonic stem cells show the same potential therapeutic effects as those derived from spinal cord and offer great promise as an unlimited source of neural stem cells for transplantation. We found that embryonic stem cell-derived neural stem cells can differentiate into motor neurons in vitro and in vivo. In addition, following their intrathecal transplantation into spinal muscular atrophy mice, the neural stem cells, like those derived from spinal cord, survived and migrated to appropriate areas, ameliorated behavioural endpoints and lifespan, and exhibited neuroprotective capability. Neural stem cells obtained using a drug-selectable embryonic stem cell line yielded the greatest improvements. As with cells originating from primary tissue, the embryonic stem cell-derived neural stem cells integrated appropriately into the parenchyma, expressing neuron- and motor neuron–specific markers. Our results suggest translational potential for the use of pluripotent cells in neural stem cell-mediated therapies and highlight potential safety improvements and benefits of drug selection for neuroepithelial cells.

Keywords: stem cells; transplantation; spinal muscular atrophy; motor neuron

Abbreviations: BDNF = brain-derived neurotrophic factor; ChAT = choline acetyl transferase; ELISA = enzyme-linked immunosorbent assay; GDNF = glial cell line-derived neurotrophic factor; GFP = green fluorescent protein; HB9 = homeobox-9; MAP2 = microtubule-associated protein 2; NT3 = neurotrophin-3; PD = postnatal day; SMI32 = neurofilament H non-phosphorylated; SMN = survival of motor neuron protein; SOX = sex-determining region Y box; TGF-α = transforming growth factor-α
Introduction

Spinal muscular atrophy, a common autosomal recessive neuromuscular disease, is caused by mutations in the survival of motor neuron 1 gene (SMN1), leading to the reduction of SMN protein expression (Lefebvre et al., 1995) and resulting in the selective degeneration of spinal cord motor neurons (Crawford and Pardo, 1996). Clinically, spinal muscular atrophy patients develop increasing weakness in all muscles, eventually including those needed for breathing. In more than half of patients, spinal muscular atrophy starts before age of 6 months (spinal muscular atrophy type 1) and typically leads to death by age of 2 years (Munsat and Davies, 1992). Disease severity is primarily under the influence of the number of SMN2 copies (Lefebvre et al., 1997). SMN2 differs from SMN1 by a single nucleotide, which results in the majority of SMN2 transcripts lacking exon 7 and encoding an unstable, truncated protein (Lorson et al., 1999). There is no cure for spinal muscular atrophy, but thus far, mouse models of the disease have provided promising insights into the mechanisms of motor neuron death and the application of potential therapeutic strategies (Schmid and DiDonato, 2007).

The existing transgenic mouse models of spinal muscular atrophy cover a spectrum from the most severe phenotype of SMN2+/+Smn−/− (Monani et al., 2000), with an average lifespan of <5 days to the SMN2+/+Smn−/-SMN2A2G/+ mice that live longer (almost a year) (Monani et al., 2003). Mice lacking the mouse Smn gene but with two copies of the human SMN2 gene and an additional SMN cDNA lacking exon 7 (SMN1.D7) (Smn−/-;SMN2A2G/+;SMN1.D7/+ mice; referred to herein as spinal muscular atrophy type phenotype, with a lifespan of ~2 weeks (Le et al., 2005). The lifespan in this spinal muscular atrophy model is sufficient to allow cell engraftment, while the phenotype is distinct and resembles the human form. This model, with a moderate lifespan accompanying easily measurable phenotypes, is potentially useful because therapeutic changes may be more readily observable than those possible with more severely or mildly affected models.

Among the therapeutic strategies for which these models hold potential is stem cell transplantation, a promising therapeutic approach for motor neuron disease. The goal is for transplanted cells to support endogenous motor neurons, through delivery of neuroprotective factors and partially by replacement of neuronal and non-neuronal cells (Hedlund et al., 2007; Koliatsos et al., 2008; Suzuki and Svendsen, 2008).

We recently reported our success with the transplantation of murine primary neural cells derived from spinal cord. These spinal-cord-derived neural stem cells ameliorated the disease phenotype in the SMN1.D7 model of spinal muscular atrophy (Corti et al., 2008), migrating into the parenchyma and generating a small proportion of motor neurons. In addition, we showed for the first time that neural stem cell transplantation positively affects the spinal muscular atrophy disease phenotype. Although these neural stem cells showed promise, their derivation from a spinal cord source presented limits in terms of further pre-clinical implementation because of scarcity within adult central nervous system tissues and other ethical and technical issues (Björklund and Lindvall, 2000).

On the other hand, pluripotent stem cells such as embryonic stem cells or induced pluripotent stem cells might represent an unlimited, phenotypically well-characterized cell source (Srivastava et al., 2008; Abeliovich and Doege, 2009). These cell sources differentiate in vitro and in vivo into neural stem cells and motor neurons (Wichterle et al., 2002; Li et al., 2005; Dimos et al., 2008; Ebert et al., 2009). What remains to be investigated is the efficacy in spinal muscular atrophy of neural stem cells derived from these pluripotent sources compared with the results with neural stem cells derived from spinal cord.

Another consideration for such transplantations is safety. Recently, concerns have arisen over the safety of the experimental therapeutic approach for neural stem cell transplantation; these concerns were triggered by reports of formation of a donor-derived brain tumour following neural stem cell transplantation in a patient with ataxia telangiectasia (Amariglio et al., 2009). The strategy we describe here targets increasing the safety of the cell source without affecting efficiency in generating functional motor neurons.

In the present study, we addressed whether neural stem cells derived from pluripotent stem cells can modify the spinal muscular atrophy phenotype in a spinal muscular atrophy mouse model as the spinal-cord-derived cells did in our previous work. In addition, we used neural stem cells from wild-type murine embryonic stem cells and drug-selectable embryonic stem cell lines. For the latter, treatment with ganciclovir and G418 will select against undifferentiated embryonic stem cells and for neuroepithelial cells, respectively, promoting neuronal differentiation and increasing cell safety. We found that after transplantation into spinal muscular atrophy mice, pluripotent-derived neural stem cells can migrate into the parenchyma, generate motor neuron cells, improve the phenotype and survival of spinal muscular atrophy mice and confer neuroprotection.

Methods

Animal models

This triple-mutant spinal muscular atrophy mouse harbours two transgenic alleles and a single targeted mutant. The Tg(SMN2 delta7)4299Ahmb allele consists of an spinal muscular atrophy cDNA lacking exon 7 whereas the Tg(SMN2)89Ahmb allele consists of the entire human SMN2 gene. Heterozygous Smn knockout mice with human SMN2 transgenes were crossed to generate transgenic mice homozygous for the knock-out Smn alleles (spinal muscular atrophy mice, SMN2+/−;Smn−/−; mSmn−/−) [line 4299; FVB.Cg-Tg(SMN2+ delta7)4299Ahmb Tg(SMN2)89Ahmb Smn−/−], The mice were genotyped using a PCR-based assay on genomic DNA from tail biopsies, as described previously (Le et al., 2005).

All transgenic animals were purchased from The Jackson Laboratory. All animal experiments were approved by University of Milan and Italian Ministry of Health review boards, in compliance with US National Institutes of Health Guidelines (Corti et al., 2006).
Embryonic stem cell culture

The embryonic stem cell wild-type line was obtained from the American Type Culture Collection. These cells were also genetically engineered to express a G418-selectable green fluorescent protein (GFP) gene reporter driven by a cytomegalovirus promoter. OSG cells (kindly provided by Prof. A. Smith) were subjected to sequential gene targeting to integrate gfp into the sex-determining region Y box (Sox)-2 locus and hygromycin-thymidine kinase into the Octamer-4 (Pou5f) locus (Billon et al., 2002). The term OSG refers to a cell line generated by sequential gene targeting to integrate hygromycin-thymidine kinase into the Octamer-4 (Pou5f) locus (O letter), bgio into the sex-determining region Y box (Sox)-2 locus (S letter) and a puromycin-selectable GFP transgene (G letter) driven by the ubiquitous CAG expression unit (Billon et al., 2002). OSG cells also carry a puromycin-selectable GFP transgene driven by the ubiquitous CAG expression unit (Billon et al., 2002). The embryonic stem cell wild-type line (ES-C57BL/6, American Type Culture Collection) and OSGs were grown and maintained in an undifferentiated state (Smith et al., 1988) on a feeder layer or 0.1% gelatin-coated dishes (Costar T-25, Becton, Dickinson) in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 20% foetal calf serum (Invitrogen), non-essential amino acids (Invitrogen), sodium pyruvate (1 mM) (Invitrogen), penicillin G (100 units/ml), streptomycin (100 mg/ml) (Invitrogen), l-glutamine (292 mg/ml) (Invitrogen), 2-mercaptoethanol (0.1 mM) and murine recombinant leukaemia inhibitory factor (1000 units/ml, Euromed). Cells were passaged every other day. Exposure of undifferentiated OSG cells to ganciclovir (2.5 mM) (Sigma–Aldrich) eliminated all cells within 4 days.

Derivation of neural stem cells from embryonic stem cells

To generate neural stem cells, embryonic stem cells were trypsinized and plated without leukaemia inhibitory factor in serum-free neurobasal medium (GIBCO, Invitrogen), supplemented with N2 and B27 (Invitrogen). After 3–4 days, cells appeared in the rosette conformation typical of neuroepithelial cells. These neuroepithelial-like cells could be isolated and propagated in neurobasal medium (plus N2, B27), epidermal growth factor and fibroblast growth factor-2 (10 ng/ml, Sigma–Aldrich). In most experiments with genetically engineered embryonic stem cells, the medium was also supplemented with G418 (100 μg/ml) and ganciclovir (2.5 μM) (Sigma–Aldrich) to select for neuroepithelial cells and against undifferentiated embryonic stem cells, respectively.

Differentiation of neural stem cells into motor neurons

For in vitro priming (Wu et al., 2002), neural stem cells were cultured as previously described (Corti et al., 2007) in neurobasal medium plus N2, 0.1 mM 2-mercaptoethanol, 20 ng/ml β-fibroblast growth factor, 1 μg/ml laminin, 5 μg/ml heparin, 10 ng/ml neural growth factor (Invitrogen), 10 ng/ml sonic hedgehog (R&D Systems), 10 μM forskolin (Sigma) and 1 μM retinoic acid (Sigma) for 48 h or 5 days. To promote a motor neuron phenotype further, glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor, insulin-like growth factor 1 and neurotrophin-3 (NT3) (10 ng/ml, R&D Systems) were added to the medium.

Immunocytochemistry on cell culture

Immunocytochemistry was performed using standard protocols, as described previously (Corti et al., 2008). The following proteins were evaluated: Sox1 (rabbit polyclonal, 1:100; AbCam), Sox2 (rabbit polyclonal, 1:100; Chemicon), Lewis X (biotin-conjugated anti-mouse prominin 1, 1:100; Bioscience), nestin (rabbit polyclonal, 1:100; Chemicon); beta III-tubulin (mouse monoclonal, 1:200; Chemicon), anti-microtubule-associated protein 2 (MAP2; mouse monoclonal, 1:100; Sigma–Aldrich), anti-choline acetyl transferase (anti-ChAT; rabbit polyclonal, 1:100; Chemicon), anti-Islet-1 (rabbit polyclonal, 1:200; Chemicon), anti-homeobox-9 (anti-HB9; rabbit polyclonal, 1:200; Chemicon), Octamer-4 (rabbit polyclonal, 1:100; Chemicon), and streptavidin Alexa Fluor 647-conjugated and mouse Alexa 488-conjugated antibodies recognizing GFP (rabbit polyclonal, 1:400; Molecular Probes). Rhodamine-conjugated bungarotoxin was purchased from Molecular Probes (1:1000). Quantitative analysis of cell phenotypes of differentiated neural stem cells was performed as described previously (Corti et al., 2008).

Western blot of cell culture

Proteins were extracted from embryonic stem cells, neural stem cells and motor neurons in cell culture as previously described (Corti et al., 2008). Blots were probed for expression of beta III-tubulin (mouse monoclonal, 1:1000; Chemicon), Islet-1 (rabbit polyclonal, 1:700; Abcam), HB9 (goat polyclonal, 1:700; Santa Cruz Biotechnology) and neurofilament H non-phosphorylated (SMI32) (mouse monoclonal, 1:1000; Covance) with β-actin (mouse monoclonal, 1:1000; Sigma) as the internal control.

Real-time reverse-transcription PCR on cell culture

Total RNA was extracted from cell culture of embryonic stem cells, neural stem cells and motor neurons using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) including DNase treatment to remove potential genomic DNA contamination. RNA quality was assessed after both amplifications using spectrophotometric and electrophoretic analysis on agarose gels.

Total RNA was reverse transcribed into complementary DNA using random hexamers and Transcripter Reverse Transcriptase (Roche Diagnostics, Indianapolis, IN, USA). Real-time PCR was performed according to the manufacturer’s protocol using TaqMan Gene Expression Assays and the ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The gene expression assays used were SOX2 (Mm00488369 s1); SOX1 (Mm00486299 s1); HB9 (Mm00658300 g1); and ChAT (anti-ChAT, Mm01221882 m1); with the values were corrected for β-actin (Mm00607939 s1). Cycle threshold (Ct) values were calculated using the ∆∆Ct method (Livak and Schmittgen, 2001). The reactions were performed in triplicate and averaged.

Neural stem cell transplantation

Before cell transplantation, neural stem cells were cultured (for priming) in neurobasal plus N2, 0.1 mM 2-mercaptoethanol, 20 ng/ml β-fibroblast growth factor, 1 μg/ml laminin, 5 μg/ml heparin, 10 ng/ml nerve growth factor (Invitrogen), 10 μM forskolin, 1000 nM sonic hedgehog (R&D Systems, Minneapolis, MN) and 1 μM retinoic acid for 5 days. On the day of transplant, the primed cells were
harvested with trypsin (0.05% trypsin/ethylenediaminetetraacetic acid) and centrifuged in Dulbecco’s modified Eagle’s medium/foetal bovine serum (1000 rpm/7 min) (Corti et al., 2007).

Murine dermal fibroblasts (primary culture from neonatal skin) were cultured as controls as described previously (Huard et al., 1998), and prepared for transplantation identically to the ‘primed’ cells as described previously (Corti et al., 2007).

One-day-old spinal muscular atrophy mice pups were used as graft recipients. A total of 20,000 cells were transplanted into the cerebrospinal fluid of cryoanesthetized animals as previously described (Corti et al., 2008). Injection of cells and use of marker dye were performed and controls managed as described in previous work (Fedorova et al., 2006).

Spinal muscular atrophy mice were divided into groups (treated: transplantation with wild-type neural stem cell ‘primed’ cells, n = 24, 12 male; OSG-neural stem cell ‘primed’ cells, n = 24, 12 male; fibroblasts, n = 24, 12 male; and untreated: vehicle-injected, n = 24, 12 male) evaluated up to the end stage for neuromuscular function, survival record and histology of donor cell phenotype. The study was designed so that littermates were distributed equally into the treated and untreated groups. Another three groups (treated and untreated spinal muscular atrophy mice and wild-type) were analysed, respectively, for histological quantification (for each group/point: n = 6; 13 days).

Assessment of survival, strength assay and open-field test

Treated and untreated spinal muscular atrophy mice were monitored daily following transplantation for clinical signs of disease. The investigators performing the functional assessment were blind to the treatment. The mice were killed at the clinical end point when they presented difficulties in feeding, a clear downward trend and breathing problems as previously described (Azzouz et al., 2004). The body weight was recorded daily.

Spinal muscular atrophy mice were used to evaluate grip strength as previously described (Grondard et al., 2005; Corti et al., 2008). Observers were blinded to all groups during the tests. The data were analysed by ANOVA followed by a Tukey’s post-hoc analysis for multiple comparisons.

Ambulatory behaviour was assessed in an open-field test as previously described (Grondard et al., 2005; Corti et al., 2008). The observer was blind to the groups.

Tissue analysis

The animals were sacrificed at the end stage of the disease, perfused and fixed with 4% paraformaldehyde in phosphate buffered saline (pH 7.4). The spinal cord was isolated, immersed in paraformaldehyde solution for 1 h, then in 20% sucrose solution in phosphate buffered saline (pH 7.4) overnight, and frozen in Tissue-Tek Optimal Cutting Temperature compound with liquid nitrogen. The spinal cords were sectioned at 40 μm thick) of the lumbar spinal cords were made, and one of every five sections was processed and Nissl stained, as reported previously (Grondard et al., 2005). The number and diameter (soma) of all motor neurons counted in these cross sections (n = 50 for each mouse) were analysed. The sections were analysed at 200× magnification in the anterior horn (either left or right) for the presence of all neurons in that region. All cells were counted within the ventral horn below an arbitrary horizontal line drawn from the central canal. Only neuronal cells showing at least one nucleolus located within the nucleus were counted, as previously described (Grondard et al., 2005).

Distal hind limbs were dissected and frozen in Tissue-Tek Optimal Cutting Temperature compound with liquid nitrogen. Sections (10 μm) were mounted on slides and stained with haematoxylin and eosin (50 sections for each animal). Digital images were captured using a Zeiss Axio Scope microscope and analysed with National Institutes of Health Image-J software (http://rsweb.nih.gov/ij/) for tibialis anterior cross-sectional area (original magnification, 5×), tibialis anterior myofibre number (original magnification, 10×) and myofibre diameter (original magnification, 40×). Myofibre diameter was determined by measuring the largest diameter of at least 300 neighbouring myofibres per animal.

Histological analysis

Serial cross sections (12 μm thick) of the lumbar spinal cords were made, and one of every five sections was processed and Nissl stained, as reported previously (Grondard et al., 2005). The number and diameter (soma) of all motor neurons counted in these cross sections (n = 50 for each mouse) were analysed. The sections were analysed at 200× magnification in the anterior horn (either left or right) for the presence of all neurons in that region. All cells were counted within the ventral horn below an arbitrary horizontal line drawn from the central canal. Only neuronal cells showing at least one nucleolus located within the nucleus were counted, as previously described (Grondard et al., 2005).

Co-culture of primary motor neurons of the spine and neural stem cells

We derived primary motor neuron cultures from E12.5 spinal muscular atrophy and wild-type mice as previously described (Corti et al., 2008). The culture medium was a motor neuron medium supplemented with a cocktail of trophic factors (Corti et al., 2008). We performed a co-culture assay to separate the primary motor neurons from the neural stem cells with a microporous membrane as previously described (Locatelli et al., 2007; Corti et al., 2008).

Axon length and growth cone measurement

Axon and growth cone analysis was performed as described previously (Rossoll et al., 2003; Corti et al., 2008). Motor neurons grown for 7 days on glass coverslips in co-culture assay were fixed with
parafomaldehyde and subsequently with acetone. The cells were incubated overnight at 4°C with primary antibodies as follows: rabbit antibodies against phospho-tau (1 μg/ml; Sigma–Aldrich), medium-weight neurofilament (1:500; Abcam) and a mouse monoclonal antibody against MAP2 (1:1000; Chemicon). Cells were then washed and incubated for 1 h at room temperature with conjugated secondary antibodies (1:200; Dako). After washing, the coverslips were mounted.

Motor neurons were scored as previously described (Corti et al., 2007). Medium was added (1.5 g/ml). Mouse Immunoglobulin G (Sigma), used at 0.5 μg/ml, was added (1.5 g/ml). Medium was added (1.5 g/ml) and 24 h later was removed for ELISA analysis (12 independent experiments for each cytokine). BDNF, GDNF, NT3 and TGF-β1 were analysed in the lumbar spinal cord (n = 6 for each condition) by ELISA. The detection of BDNF, GDNF and NT3 was performed as previously described (Chen et al., 2007). Medium was added (1.5 x 10⁶ cells, 1.5 ml medium) and 24 h later was removed for ELISA analysis (12 independent experiments for each cytokine). BDNF, GDNF, NT3 and TGF-β1 cytokines were analysed in the lumbar spinal cord (n = 6 for each condition) by ELISA. The detection of BDNF, GDNF and NT3 cytokines was performed following manufacturer instructions (Promega). The TGF-β1 ELISA in vivo was performed as previously described (Chen et al., 2007).

Neutralizing antibodies

Incubation of primary motor neurons and primed neural stem cell co-cultures with the following neutralizing antibodies was performed with anti-TGF-β1 antibody (Calbiochem), anti-BDNF (Calbiochem), anti-GDNF (R&D Systems), anti-NT3 (R&D Systems) and control mouse Immunoglobulin G (Sigma), used at 0.5 μg/ml.

Statistical analysis

The Kaplan–Meier survival analysis and the log-rank test were used for survival comparisons. The growth curve, assay of strength and open-field tests were analysed by ANOVA followed by a Tukey’s post-hoc analysis for multiple comparisons. Numbers and size of motor neurons as well as muscle and neuromuscular junction data were statistically evaluated by one-way ANOVA followed by a Tukey’s post-hoc analysis. The statistical analysis of real-time reverse-transcription PCR data was performed using Student’s t-tests. For cytokine detection and for co-culture assays, differences between means were analysed using two-tailed Student’s t-tests. We used StatsDirect for Windows (version 2.6.4) for all analyses, and the null hypothesis was rejected at the 0.05 level.

Results

Efficient differentiation of murine embryonic stem cells into adherent neural stem/precursor cells can generate motor neurons in vitro

To study the neural differentiation potential of murine embryonic stem cells in vitro, we used wild-type embryonic stem cells as well as a drug-selectable cell line, the OSG cells (OSG-embryonic stem cells) (Billon et al., 2002). We applied a multistage differentiation protocol previously developed to promote the conversion of murine embryonic stem cells into neural stem cells (Ying et al., 2003).

These culture conditions resulted in the emergence by 3–4 days of rosette conformations typical of neuroepithelial cells. After 7 days, 67.6 ± 5.6% of wild-type embryonic stem cells expressed Sox1 (n = 15) (data not shown). The neuroepithelial-like cells were then isolated and propagated in media containing epidermal growth factor and fibroblast growth factor-2 in adherent conditions. These cells homogeneously expressed neural stem cell marker proteins such as Sox1, Sox2, Lewis X and nestin (>95%) (Fig. 1). Under differentiation conditions, 62.4 ± 5.1% of the cells generated MAP2-positive neurons with extended neuronal processes (n = 15) (data not shown).

We then tested whether wild-type embryonic stem cell-derived neural stem cells can give rise to clinically relevant neuronal subtypes such as ventral spinal cord motor neurons. Following the protocols developed for mouse embryonic stem cells, we treated embryonic stem cell-derived neural precursor cells with the regional patterning factors sonic hedgehog and retinoic acid, with the addition of neurotrophins. Quantification of the positive cells revealed that the number of ChAT-positive neurons was 28.6 ± 4.6% (n = 15 independent experiments). These cells also expressed other specific motor neuron markers (Islet-1, HB9, SMI32) as demonstrated by immunocytochemistry, western blot and real-time reverse-transcription PCR (motor neurons versus neural stem cells, P < 0.00001) (Figs. 2, Supplementary Figs. S1 and S2). ChAT and SMI32 have been used as motor neuron markers in several studies (Clement et al., 2003; Suzuki et al., 2007). Quantification analysis revealed that the number of cells double positive for ChAT and HB9 was 27.9 ± 4.7% (n = 15 independent experiments) in wild-type embryonic stem cells.

To increase the differentiation rate into neural stem cells and increase their safety, we used OSG-embryonic stem cells that are genetically engineered to express the hygromycin-thymidine kinase fusion gene from the Octamer-4 locus and the β-geo gene from the Sox2 locus (Billon et al., 2002). Treatment of the doubly targeted embryonic stem cells with both ganciclovir and G418 selected against undifferentiated embryonic stem cells and for neuroepithelial cells, promoting neuronal differentiation. Three days following the removal of leukaemia inhibitory factor, we treated these doubly targeted embryonic stem cells for 4 days with G418 and ganciclovir. Following this treatment, no cells expressing Octamer-4 were detectable (data not shown), suggesting that no residual embryonic stem cells were present. In contrast, 88.5 ± 6.3% of the cells expressed Sox1 (n = 15; OSG-embryonic stem cells versus wild-type embryonic stem cells, P < 0.00001) as well as Sox2 and nestin, indicating a significant enrichment in neuroepithelial cells compared with wild-type embryonic stem cells. These neuroepithelial-like cells were then isolated and propagated in media containing epidermal growth factor and fibroblast growth factor-2 in adherent conditions. They homogeneously expressed neural stem cell marker proteins such as nestin, Sox1, Sox2 and Lewis X (>95%) (Supplementary Fig. S3). Real-time reverse-transcription PCR analysis confirmed the upregulation of Sox1 and Sox2 in OSG-neural stem cells compared with undifferentiated OSG-embryonic stem cells (neural
stem cells versus embryonic stem cells, $P < 0.00001$) (Supplementary Fig. S3).

Under differentiation conditions, the percentage of the resulting MAP2-positive neurons was $73.5 \pm 4.2\%$, significantly higher compared with those resulting from wild-type embryonic stem cells ($62.4 \pm 5.1\%$) ($n = 15$; OSG-embryonic stem cells versus wild-type embryonic stem cells, $P < 0.00001$) (data not shown).

Using the same differentiation protocols as described for the wild-type embryonic stem cells, we tested whether engineered embryonic stem cell-derived neural stem cells can differentiate into motor neurons. Indeed, $37.7 \pm 3.3\%$ of motor neuron-like cells (ChAT-positive cells) and $37.5 \pm 3.5\%$ of motor neuron cells double positive for Hb9 and ChAT were generated from OSG-embryonic stem cells, significantly higher compared with the percentage generated from the wild-type embryonic stem cells ($n = 15$; $P < 0.00001$) (Supplementary Fig. S2). These cells were also positive for other specific motor neuron markers, as demonstrated by immunocytochemistry and western blot (Supplementary Fig. S4).

Embryonic stem cell-derived neural stem cells migrate extensively into the spinal muscular atrophy spinal cord and differentiate into neurons and motor neurons after transplantation in the cerebrospinal fluid

The wild-type embryonic stem cells and OSG-embryonic stem cells were engineered to express the GFP gene reporter for easy detection of embryonic stem cell-derived neural stem cells. To explore their engraftment capacity, we intrathecally transplanted 20 000 neural stem cells derived from the GFP-positive embryonic stem cells into spinal muscular atrophy mice at one day of age [postnatal day (PD) 1]. The transplantation protocol and cell concentration were fixed based on previous results obtained from transplantation of primary neural stem cells into spinal muscular atrophy mice (Corti et al., 2008).
Controls were Smn+/+ SMN2+/+ SMN1/7 mice (wild-type for SMN locus) and transgenic spinal muscular atrophy littermates of treated mice, which received only the vehicle. To limit possible bias, siblings were distributed equally throughout the treated and control groups and equally divided between male and female.

Analysis of transplanted mice (and control groups) was performed at the end stage of the disease. Transplanted cells formed perimeningeal clusters, and some migrated extensively into the host spinal cord tissue. GFP-positive cells were found along the rostro-caudal extension in the spinal cord within both the cervical and lumbar enlargement. The highest densities of transplanted cells were found in the grey matter, particularly in the anterior horns. Unbiased stereological quantification with optical dissectors and random sampling of GFP-engrafted cells demonstrated that 2578 ± 212 cells were present in the spinal cord parenchyma that was transplanted with wild-type embryonic stem cells and 2976 ± 215 with OSG-embryonic stem cells (OSG versus wild-type, P < 0.00001).

To evaluate if transplanted cells acquired a neuronal or non-neuronal phenotype, we performed confocal immunohistochemical analysis for neuroectodermal antigens. Integrated cells displayed various complex neuronal and glial morphologies expressing the neuronal marker proteins NeuN and β-III-tubulin, neurofilament or the glial fibrillary acidic protein (Figs. 3, Supplementary Fig. S5 and S6). The predominant phenotype of transplanted cells was neuronal, as demonstrated by the double-positive staining for the neuronal-specific antigen MAP2 and GFP (46.8 ± 4.9% in wild-type embryonic stem cells and 49.8 ± 4.4% for OSGs; data not shown). Immunohistochemical analysis for glial antigens (glial fibrillary acidic protein and O4) combined with GFP showed the presence of 22.5 ± 4.1% astrocytes in wild-type embryonic stem cells and 21.3 ± 3.6% in OSG-embryonic stem cells. Furthermore, 3.4 ± 1.2% oligodendrocytes were detected in the wild-type group and 3.3 ± 1.1% in the OSG group. In addition, a fraction of these cells presented the features of neural stem cell/progenitor cells, expressing both nestin and GFP (23.4 ± 3.2% in wild-type embryonic stem cells

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**Figure 2** Wild-type embryonic stem cell-derived neural stem cells can differentiate into motor neurons in vitro. (A) Wild-type embryonic stem cells (ESC) (GFP, green) differentiated into neural stem cells (NSC). (B) Merge of GFP (green), nestin (red) and Lewis X (light blue). (C) Wild-type embryonic stem cell-derived neural stem cells then differentiated into motor neurons (merge of GFP, green, and ChAT, red). Single images of the merged pictures (B and C) are shown in supplementary files (Fig. S1). (D) GFP-labelled motor neuronal cells. (E) Same cells positive for ChAT. (F) Merge of D and E. (G) GFP-labelled motor neuronal cells. (H) Same cells positive for Islet-1. (I) Merge of G and H. (J) Western blot analysis confirmed the expression of neuronal (beta III-tubulin) and motor neuronal markers (Islet-1, HB9, SMI32) in terminally differentiated motor neurons (MN) compared with neural stem cells; β-actin was internal control. (K) Confirmation via real-time reverse-transcription PCR of upregulation in motor neurons of the motor neuronal marker ChAT. (L) Confirmation via real-time reverse-transcription PCR of upregulation of the motor neuronal marker HB9 in motor neurons. For K and L, upregulation was significant in motor neurons compared with neural stem cells (P < 0.00001). Scale bars: A–F = 75 μm; G–I = 50 μm. LIF = leukaemia inhibitory factor; EGF = epidermal growth factor; FGF = fibroblast growth factor; Shh = sonic hedgehog; RA = retinoic acid.
and 21.1 ± 3.3% in OSG-embryonic stem cells; data not shown). Finally, a fraction of transplanted cells differentiated into motor neurons, as suggested by their morphology and reactivity for motor neuron-specific markers, including ChAT and SMI32 (Figs. 3 and Supplementary Fig. S5).

Unbiased stereological quantification with optical dissectors and random sampling based on (i) ChAT and GFP double immunoreactivity, (ii) the position within the anterior horns and (iii) the cell size (soma diameter > 25 μm; Beers et al., 2006) demonstrated that the number of ChAT-GFP cells was 356 ± 27 cells per spinal cord in the wild-type embryonic stem cell group and 399 ± 28 cells in the OSG-embryonic stem cell group (OSG versus wild-type, P < 0.0001).

Donor-derived cells extended their axons horizontally into the white matter, and some cells also extended processes into the ventral roots (a mean of 32 ± 12 GFP-positive axons per animal in wild-type embryonic stem cells and 43 ± 13 in the OSG-embryonic stem cells; OSG versus wild-type, P < 0.00001), suggesting that they can elongate their axons toward the periphery (Fig. 3I–K). GFP axon length was estimated to be 3–5 mm distal to the root entry into the spinal cord.

Throughout the study, we observed no teratoma formation either within the wild-type embryonic stem cell group or the OSG group. To evaluate the safety of genetically modified OSG-embryonic stem cells further, we assessed cell death and elimination in vivo following tumour formation in a whole animal under ganciclovir treatment. For this purpose, we injected neonatal mice intrathecally with the undifferentiated OSG-embryonic stem cells (1 × 10^6 cells; n = 20). After 1–2 months, four mice developed teratomas. One mouse was used as a control and was injected daily with saline, whereas the three remaining mice were injected daily with 50 mg/kg ganciclovir for 10 days. During this period, tumour mass was reduced by more than 70%, while one of the tumours disappeared completely.

At the time of writing, the follow-up period for wild-type mice, transplanted with wild-type neural stem cells and OSG-neural stem cells, has reached 12 weeks after transplantation. At this time point, we have detected surviving transplant-derived cells...
that present a neuronal and motor neuronal phenotype within the host spinal cord. We have observed no formation of tumours up to this time. Representative pictures of wild-type spinal cord and the motor neuronal phenotype are presented in the Supplementary data (Fig. S7).

Functional recovery follows transplantation of embryonic stem cell-derived neural stem cells

Our next goal was to establish whether improvement of the spinal muscular atrophy phenotype could be achieved through injection of embryonic stem cell-derived neural stem cells. The general condition, weight and motor function of the animals were tested daily. The treated mice displayed a higher survival rate in comparison to untreated mice (log-rank test; wild-type embryonic stem cell group versus untreated, $\chi^2 = 51.88, P < 0.00001$; OSG group versus untreated, $\chi^2 = 53.18, P < 0.00001$) (Fig. 4). In fact, compared with the wild-type embryonic stem cell group, the mouse group transplanted with OSG-embryonic stem cells showed greater improvement in survival (log-rank test; wild-type embryonic stem cell group versus untreated, $\chi^2 = 6.4, P = 0.011$).

Mean survival was 12.87 ± 1.82 days ($n = 24$) in untreated mice, 19.62 ± 1.88 days in wild-type embryonic stem cells, and 21.04 ± 1.83 days in OSG-embryonic stem cell-treated mice ($n = 24$). Therefore, survival was prolonged by 6.75 days in the wild-type embryonic stem cell group and by 8.17 days in the OSG group, interpreted as an increase of 52.44 and 63.48%, respectively, over the lifetime.

The mean survivals achieved with embryonic stem cell-derived neural stem cells, especially for the OSG group, are greater than those we previously observed with primary neural stem cells (18.16 ± 1.78 days in neural stem cell-treated mice, $n = 24$, representing a gain of 39.26% over the lifetime with respect to their controls) (Corti et al., 2008).

The maximum survival period observed in the OSG-embryonic stem cell-treated mice was 24 days; it was 16 days for the untreated mice. Fig. 4B shows the Kaplan–Meier survival curves. Male and female mice showed no statistically significant difference in survival rate (data not shown).
We also evaluated the survival curve for spinal muscular atrophy mice transplanted with murine primary fibroblasts (Fig. 4B) and found that spinal muscular atrophy mice transplanted with embryonic stem cell-derived neural stem cells survived longer than those grafted with primary fibroblasts (fibroblasts: 13 ± 2.15 days; wild-type embryonic stem cells versus fibroblasts, $\chi^2 = 44.31$, $P < 0.00001$; OSG-embryonic stem cells versus fibroblasts, $\chi^2 = 49.9$, $P < 0.00001$).

One of the first clinical symptoms in spinal muscular atrophy mice is body weight reduction. By five days of age, untreated spinal muscular atrophy mice presented significant weight differences compared with wild-type and treated mice, differences that persisted throughout life. Treated mice had a markedly greater increase in body weight, significantly different at 13 days of age compared with untreated animals, with the OSG group presenting a greater weight with respect to the other groups (ANOVA and Tukey’s; wild-type embryonic stem cell mice and 17.14 ± 2.91% in OSG mice, while spinal muscular atrophy untreated animals presented a reduction of 38.9 ± 2.96% ($P < 0.00001$). Because the stereological count based on ChAT and GFP immunoreactivity indicated about 6–7% of donor cells in the two treated groups, these data demonstrate strikingly that neural stem cell transplantation had a neuroprotective effect on endogenous motor neurons.

Moreover, we analysed the size of these neurons; the mean diameter of neurons was 36.3 ± 3.18 μm in wild-type littermates, 31.1 ± 3.53 μm in untreated spinal muscular atrophy mice, 34.9 ± 3.47 μm in treated wild-type embryonic stem cell spinal muscular atrophy mice and 35.3 ± 3.21 μm in OSG-embryonic stem cell-treated mice (Fig. 5) ($P < 0.00001$).

One of the major characteristics of the spinal muscular atrophy phenotype is muscle atrophy. Compared with wild-type littermates, the mean total cross-sectional area, the diameter of myofibres and the number of myofibres in the tibialis anterior muscle of the untreated spinal muscular atrophy mice were significantly reduced (Fig. 6). Compared with untreated mice, we found a significant increase in total muscle area ($P < 0.00001$), in mean myofibre diameter ($P < 0.00001$) and in myofibre number ($P < 0.00001$) following neural stem cell transplantation in both the wild-type embryonic stem cell and OSG-embryonic stem cell groups (Fig. 6).

These data are in line with what we have previously observed in experiments describing primary neural stem cell transplantation, and they confirm that neuroprotection is a key factor in the beneficial effect of neural stem cell transplantation in spinal muscular atrophy mice.

### Neural stem cell transplantation provides support to spinal muscular atrophy motor neurons and their muscle connections

To investigate motor and survival improvement in spinal muscular atrophy-transplanted mice, we performed histological analysis of muscle and spinal cord tissues at PD13 in a cohort of mice (spinal muscular atrophy wild-type embryonic stem cell-treated mice, $n = 6$; spinal muscular atrophy OSG-embryonic stem cell-treated mice, $n = 6$; spinal muscular atrophy untreated mice, $n = 6$; and wild-type littermates, $n = 6$).

We analysed the number and size of motor neurons within the ventral horn of the lumbar tract. Compared with wild-type littermates, the number of motor neurons was significantly reduced in spinal muscular atrophy mice ($P < 0.00001$), and the morphology differed. Transplantation of neural stem cells gave rise to an average of 7.53 ± 1.33 ventral horn neurons per horn and per section in spinal muscular atrophy wild-type embryonic stem cell-treated mice and 7.88 ± 1.34 in spinal muscular atrophy OSG-embryonic stem cell-treated animals; these values were 9.51 ± 1.38 in wild-type mice and 5.81 ± 1.36 in spinal muscular atrophy untreated animals (Fig. 5).

With respect to wild-type, the reduction of motor neurons at 13 days of age was only 20.82 ± 3.68% in wild-type embryonic stem cell mice and 17.14 ± 2.91% in OSG mice, while spinal muscular atrophy untreated animals presented a reduction of 38.9 ± 2.96% ($P < 0.00001$). Because the stereological count based on ChAT and GFP immunoreactivity indicated about 6–7% of donor cells in the two treated groups, these data demonstrate strikingly that neural stem cell transplantation had a neuroprotective effect on endogenous motor neurons.

Moreover, we investigated neuromuscular function in the animals using the grip assay and open-field test. At 13 days of age, treated mice demonstrated stable performance on the grip assay for a few seconds, while untreated mice were unable to perform the task (ANOVA and Tukey’s; wild-type embryonic stem cells versus untreated, $P < 0.00001$; OSG versus untreated, $P < 0.00001$; OSG versus wild-type embryonic stem cell, $P < 0.00001$).

We also investigated neuromuscular function in the animals using the grip assay and open-field test. At 13 days of age, treated mice demonstrated stable performance on the grip assay for a few seconds, while untreated mice were unable to perform the task (ANOVA and Tukey’s; wild-type embryonic stem cells versus untreated, $P < 0.00001$; OSG versus untreated, $P < 0.00001$) (Fig. 4D).

Untreated mice also had compromised responses to surface righting and presented with severely impaired locomotor activity and exploratory behaviour (Supplementary Movies 1–3). As demonstrated by the number of crossings, the treated spinal muscular atrophy mice maintained some locomotor activity and showed some exploratory behaviour (13 days treated versus untreated, ANOVA and Tukey’s, $P < 0.00001$) (Supplementary Fig. S8).

### Embryonic stem cell-derived neural stem cells produce neuroprotective factors

To address the molecular mechanisms underlying the improved phenotype of spinal muscular atrophy mice, we used ELISA to analyse the potential of neural stem cells to secrete neuroprotective factors in vitro.

After comparing primed neural stem cell-derived cells to the other cell types (astrocytes and fibroblasts), we found that neural stem cells secreted significant amounts of GDNF (wild-type embryonic stem cells: 752.5 ± 44.66 pg/ml; wild-type embryonic stem cells versus other cell types, $P < 0.00001$; BDNF (544.7 ± 44.66 pg/ml; wild-type embryonic stem cells versus other cell types, $P < 0.00001$; TGF-α (123.32 ± 8.76 pg/ml; wild-type embryonic stem cells versus other cells, $P < 0.00001$) and NT3 (163.34 ± 16.45 pg/ml, wild-type embryonic stem cells versus other cell types, $P < 0.00001$) (Fig. 7A–D).

Moreover, to assess further the impact of neural stem cells on spinal muscular atrophy pathogenesis, we used a co-culture system with a bottom layer of primary motor neurons from spinal muscular atrophy mice and a top layer of neural stem
cells seeded onto a microporous membrane, allowing diffusion of substances between the higher and lower compartments.

Motor neurons were fixed after 7 days in culture and immuno-stained with antibodies against MAP2 and phospho-tau to determine neurite length. The results confirm previous findings on axonogenesis defects (Jablonka et al., 2007). In fact, spinal muscular atrophy primary motor neurons alone presented a significant reduction in axon length with respect to wild-type primary motor neurons (210.6 ± 23.1 μm versus 310.5 ± 27.2 μm, P < 0.00001). When spinal muscular atrophy primary motor neurons were co-cultured in the presence of neural stem cells, this phenotype was partially rescued (Fig. 7E).

Finally, we measured the size of axonal growth cones using phospho-tau staining. Growth cones of SMN-deficient motor neurons were significantly smaller than those of wild-type motor neurons (spinal muscular atrophy: 15.1 ± 3.6 μm versus 47.1 ± 4.6 μm, P < 0.00001); notably, we observed an increase in growth cone size after co-culture of motor neurons and neural stem cells (wild-type embryonic stem cell: 25.6 ± 3.2; 27.1 ± 3.6 treated versus untreated spinal muscular atrophy mice, P < 0.00001) (Fig. 7F).

Our hypothesis is based on the fact that upregulation of neurotrophin expression may contribute to the differential axonal length enhancement seen in co-culture. To confirm this.

Figure 5 Neural stem cell transplantation increases motor neuron size and number. Spinal muscular atrophy mice were treated with vehicle (n = 6) or neural stem cells (n = 6); wild-type littermates were treated with vehicle (n = 6) on PD1. Cross-section of Nissl-stained lumbar spinal cord of (A) spinal muscular atrophy (SMA) untreated mice; (B) wild-type embryonic stem cell-neural stem cell-treated (TrwtESC) mice; (C) spinal muscular atrophy OSG-embryonic stem cell-neural stem cell-treated (TrOSG-ESC) mice; and (D) wild-type (wt) mice. (E) Mean motor neuron primary number in spinal muscular atrophy mice was lower compared with wild-type littermates and was significantly increased by neural stem cell transplantation (P < 0.00001 treated versus untreated at 13 days). (F) Mean ventral horn neuron size was smaller in spinal muscular atrophy mice compared with wild-type mice and was increased by neural stem cell transplantation (P < 0.00001). Data represent mean motor neuron number and size values ± SD at PD13. Scale bar: A–D = 200 μm.
Neural stem cell transplantation ameliorates muscle innervation in spinal muscular atrophy mice. Spinal muscular atrophy (SMA) mice were treated with vehicle ($n=6$), wild-type neural stem cells (Trwt-ESC; $n=6$) or OSG-neural stem cells (Trwt-OSG; $n=6$); wild-type (wt) littermates were treated with vehicle ($n=6$) on PD1. (A–D) Haematoxylin and eosin stained cross sections of tibialis anterior muscle. (E–H) Histograms of myofibre diameters. (I) Mean tibialis anterior muscle cross-sectional area was reduced in spinal muscular atrophy mice compared with wild-type littermates ($^* P < 0.0001$) and increased after neural stem cell transplantation ($^* P < 0.00001$). Data represent mean values ±SD. (J) Mean tibialis anterior muscle total myofibre number was reduced in spinal muscular atrophy mice compared with wild-type littermates ($^* P < 0.00001$) and increased after neural stem cell treatment ($^* P < 0.0001$). Scale bar: A–D = 200 μm.
Neural stem cells ameliorate the phenotype of primary spinal muscular atrophy motor neurons in a co-culture assay. The amounts of (A) GDNF, (B) BDNF, (C) TGF-α and (D) NT3 secreted by neural stem cells. (E) Average length of axons and (F) average growth cone area of motor neurons from spinal muscular atrophy, motor neurons from spinal muscular atrophy in co-culture with wild-type embryonic stem cells (SMATr-wtESC), motor neurons from spinal muscular atrophy in co-culture with OSG (SMATr-OSG) and motor neurons from wild-type (wt) mice. Motor neurons from spinal muscular atrophy mice co-cultured with neural stem cells exhibited a significant increase in axon length and growth cone with respect to motor neurons of the untreated animals ($^*P<0.00001$). (G) The average axonal length of motor neurons after cytokine neutralization from spinal muscular atrophy mice in co-culture with wild-type embryonic stem cell and (H) average axonal length of motor neurons from spinal muscular atrophy in co-culture with OSG.
versus control, et al. spinal cord-derived neural stem cells (Corti et al. 2009). We recently reported that primary murine neural stem cell transplantation can ameliorate the disease phenotype in a spinal muscular atrophy mouse model (Corti et al., 2008). These results demonstrated a significant reduction in neural cell death. We observed these results in primary neural stem cell-treated animals when compared with wild-type embryonic stem cell-treated animals and the mean number of donor-derived motor neurons were significantly different compared with the control (P = 0.00001; TGF-α versus control, P = 0.00004; NT3 versus control, P < 0.00001) (Fig. 7G and H).

On the basis of these findings, we investigated the expression of neurotrophins in transplanted spinal muscular atrophy spinal cord compared with untreated and fibroblast-treated spinal cord by ELISA. Analysis of spinal cord at PD 13 indicated that cell transplantation significantly increased the levels of BDNF, TGF-α and NT3 compared with their levels in vehicle-injected and fibroblast spinal muscular atrophy controls (P < 0.05) (Supplementary Fig. S9).

These data suggest that neural stem cells might exert a neuroprotective and axogenic therapeutic effect by producing growth factors.

**Discussion**

We and others have previously demonstrated that neural stem cell transplantation may have a beneficial effect on motor neuron disease phenotypes in mouse models (Corti et al., 2006, 2007; Ebert et al., 2009; Dimos et al., 2008). Growing evidence indicates that this effect is likely to be mediated through cell replacement as well as through neuroprotection of host motor neurons by numerous factors released from the donor cells (Ebert et al., 2009). We recently reported that primary murine neural stem cell transplantation can ameliorate the disease phenotype in a spinal muscular atrophy mouse model (Corti et al., 2008). These results demonstrated for the first time the therapeutic potential of neural stem cell transplantation for spinal muscular atrophy. We show in the current work that embryonic stem cell-derived neural stem cells can differentiate into motor neurons in vivo, and when transplanted into the cerebrospinal fluid can migrate along the spinal muscular atrophy spinal cord and home-in on appropriate areas, generating motor neuron-like cells. Transplantation of embryonic stem cell-derived neural stem cells significantly improved the neurological phenotype and survival of spinal muscular atrophy mice. The use of OSG cells, cells genetically modified to select motor neurons positively and select against residual embryonic stem cells, for therapy. For them to be safe, however, requires selection for the cell type of interest and against any residual embryonic stem cells. These latter are responsible for tumour formation in the recipient (Brüstle et al., 1997; Amaraglio et al., 2008).

Related to these issues of translational safety, a major finding of this study is that very similar results were obtained using different cell sources, two embryonic stem cell lines and primary neural stem cells, strengthening the potential of this cellular therapeutic strategy. Indeed, the present study not only demonstrates the success of this approach but also a potential superiority for the drug-selected cells. Whether we compare the results with the OSG-derived cells to those derived without drug selection or to those derived from primary tissue in our previous work (Corti et al., 2008), the neural stem cells derived from the drug-selected line produced superior results. We hypothesize that although the embryonic stem cell types yielded neuroepithelial cells with a similar efficiency, the genetic modification induced a more efficient generation of terminally differentiated neurons and motor neurons in vivo and in vitro.

The results of the current work also compare favourably with our previous findings using primary neural stem cells in terms of engraftment efficiency, cell differentiation and spinal muscular atrophy mice survival. In fact, both the mean number of donor cells and the mean number of donor-derived motor neurons were statistically higher in the spinal cord of OSG-neural stem cell-treated animals when compared with wild-type embryonic stem cell-treated mice. Furthermore the OSG-NCS-treated animals survived longer than the wild-type embryonic stem cell-treated group (11.04%) and longer than the neural stem cell-treated animals in our previous report (24.22%). Although neural stem cell transplantation was not sufficient to rescue the spinal muscular atrophy phenotype fully, the behavioural and survival improvement was evident. The neurological amelioration was associated with a significant change in spinal cord pathology, and the motor neuron count in treated compared with untreated mice demonstrated a significant reduction in neural cell death. We observed the generation of a lower proportion of donor-derived motor neurons, as evaluated by immunopathological characterization,
which were correctly located in the anterior horns, elongating their axons into the anterior roots; thus, we concluded that neurogenesis had a minor impact on functional recovery.

We also observed an increase in the number and size of myofibres as well as in the size of end-plates in treated spinal muscular atrophy mice compared with untreated littermates. Interestingly, the transplanted mice presented ameliorated muscle pathology and increased neuromuscular junction size, suggesting improved survival and integrity of the entire motor unit.

Overall, the functional improvement in the spinal muscular atrophy mouse models was supported by result for both neuromuscular function tests (e.g. grip assay and open-field test) and survival. Neural stem cell transplantation extended survival by 40% compared with the untreated spinal muscular atrophy group. This effect was cell specific, not having been observed following fibroblast transplantation. The increased survival time we observed with stem cell transplantation, though limited, is relevant considering previously reported results of gene therapy experimental trials using spinal muscular atrophy mice. One trial, based on the gene transfer of cardiotoxin-1 through intramuscular injection of adenoviral vectors, induced an extension of the lifespan by 30% in another mouse model (Lesbordes et al., 2003). Another, based on multiple single injections of a lentiviral vector expressing SMN in various muscles, restored SMN in motor neurons and increased life expectancy by an average of 3 and 5 days (20% and 38%), compared with LacZ and untreated animals, respectively (Azzouz et al., 2004). Another recent study involved development of a steric block antisense oligonucleotide that blocked an intronic splice suppressor element and enhanced SMN2 exon 7 inclusion, which improved the phenotype in a mouse model of spinal muscular atrophy but without detectable effects on survival (Williams et al., 2009). Delivery of bifunctional RNAs targeting an intronic repressor increased SMN levels and extended median survival in a severe model of spinal muscular atrophy, but only by one day (Baughan et al., 2009). Among pharmacological treatments, histone deacetylase inhibition with trichostatin A increased survival by 5 days (Avila et al., 2007). The combination of trichostatin A delivered early in life and with aggressive nutritional support (a complex protocol of mice caring) can result in a more sustained amelioration of the spinal muscular atrophy phenotype (Narver et al., 2008). In the context of these previous results, the 40% extension of survival in the treated mice in the current study is noteworthy.

Similarly, the neuromuscular function test results in this study must be considered in a severity context. Treated animals were able to perform the grip task stably for a few seconds, while untreated animals could not perform the task at all. In addition, treated mice show ameliorations of the compromised surface righting responses as well as the impaired locomotor and exploratory behaviours of untreated animals. The amelioration was not complete, but it was comparatively substantial.

Obviously, questions remain, some focusing on specific aspects of this work. These results were obtained by injecting 20 000 cells. Previous work has led us to estimate that this amount of cells is the more effective and well-tolerated cell concentration that can be applied via intrathecal injection in the PD1 mouse (Corti et al., 2007, 2008). Several technical constraints currently limit the amount of transplantable cells, and improvements in transplantation technique may modify this number and experimental outcomes.

Other considerations focus on the choice of a cell source. In this study, we investigated embryonic stem cell-derived neural stem cells primed toward the motor neuron phenotype. In our previous set of experiments using primary cells, we demonstrated the advantage of using primed cells with respect to undifferentiated ones (Corti et al., 2008). In contrast, terminally differentiated motor neurons would have required a direct intraparenchymal injection instead of minimally invasive intrathecal transplantation. Moreover, neural stem cells appear to exert both a cell-replacement (substituting more than one cell type) and a neuroprotective action, and also migrate extensively over the spinal cord. A comparative analysis between these two (or other) different experimental protocols is unavailable at the present time. In migrating extensively throughout the spinal muscular atrophy spinal cord, the intrathecally transplanted neural stem cells spread along both the cervical and lumbar spinal cord tracts. Our data demonstrate that the spinal muscular atrophy spinal cord presents intrinsic microenvironmental signals that may promote the engraftment and differentiation of embryonic stem cell-derived neural stem cells toward a motor neuronal phenotype in a newborn mouse. The results also suggest that neural stem cells can respond to conditions of motor neuronal ‘depletion’ after appropriate induction with neurogenetic factors.

Another consideration is gene and protein profiling. In our previous work, we investigated the molecular events that occurred in spinal muscular atrophy motor neurons after cell transplantation, performing global gene expression profile analysis on isolated endogenous motor neurons (Corti et al., 2008). We detected a number of transcripts with significant differential expression in the lumbar spinal cord motor neurons of treated and untreated spinal muscular atrophy compared with wild-type mice. We also demonstrated variation in the level of genes involved in RNA metabolism. This observation is in agreement with the role that SMN plays in RNA processing (Lefebvre et al., 1997). The modification of the gene expression profile after cell transplantation appears also to be present in this experiment with embryonic stem cell–neural stem cell treatment, as suggested by the similar characteristics of the cells used and by the very similar results obtained in vitro and in vivo. However, for this data set, we did not perform specific profile analyses to address this point.

Because cell engraftment requires time for migration, maturation and engraftment, our strategy probably cannot act effectively in the critical early stages (1–3 days of age) in this spinal muscular atrophy model, an issue that may require further elaboration. Indeed, increased efficiency of engraftment and of donor cell motor neuronal differentiation might improve the overall efficacy even in the early stage. One possible engraftment strategy is to increase the homing toward the host spinal cord. In previous work, we investigated the use of chemokine stromal cell-derived factor-1 to stimulate the migration and engraftment of neural stem cells injected systemically from the blood circulation to the central nervous system (Corti et al., 2005). Similar strategies can be investigated, and some information might be derived from the
comparison of the migratory capacity of neural stem cells derived from embryonic stem cells and primary cells detecting cell surface molecules like integrins or secreted enzymatic proteins that can digest matrix, thus influencing the migration across the meninges and in the parenchyma.

The differentiation into motor neurons is another crucial point that, based on our results, depends on priming protocols and on the characteristics of the starting cell population. The priming protocol might be improved using new agonists of sonic hedgehog pathways (Wada et al., 2009). The data obtained with OSG cells also indicate that the differentiation into motor neurons might be increased when the neural stem cell phenotype is stable and reinforced. Combined treatment of cell transplantation with drug or gene therapy might increase the therapeutic efficacy to a prospectively clinical significant level.

In conclusion, this report provides evidence that neural stem cells derived from pluripotent cells are a potentially useful therapeutic tool to restore functionality in spinal muscular atrophy.

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Supplementary material

Supplementary material is available at Brain online.

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