Transplanted ALDH hiSSClo Neural Stem Cells Generate Motor Neurons and Delay Disease Progression of nmd Mice, an Animal Model of SMARD1

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ABSTRACT

Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is an infantile autosomal recessive motor neuron disease, caused by mutations in the Immunoglobulin µ-binding protein 2 (IGHMBP2).

We investigated the potential of a spinal cord neural stem cell population isolated on the basis of aldehyde dehydrogenase activity (ALDH) to modify disease progression of nmd mice, an animal model of SMARD1.

ALDH^{hi}SSC^{lo} stem cells are self-renewing and multipotent and when intratechally transplanted in nmd mice generate motor neurons properly localized in the spinal cord ventral horns.

Transplanted nmd animals presented delayed disease progression, sparing of motor neurons and ventral root axons and increased life-span.

To further investigate the molecular events responsible for these differences, microarray and Real time RT-PCR analysis of wild-type, mutated and transplanted nmd spinal cord were undertaken.

We demonstrated a down-regulation of genes involved in excitatory amino acid toxicity and oxidative stress handling, as well as an up-regulation of genes related to the chromatin organization in nmd compared to wild-type mice, suggesting that they may play a role in SMARD1 pathogenesis. Spinal cord of nmd transplanted mice expressed high transcript levels for genes related to neurogenesis such as Doublecortin (DCX), LIS1 and drebrin. The presence of DCX-expressing cells in adult nmd spinal cord suggests that both exogenous and endogenous neurogenesis may contribute to the observed nmd phenotype amelioration.
INTRODUCTION

Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is an autosomal recessive form of infantile motor neuron disease characterized by early-onset life-threatening respiratory distress due to diaphragmatic paralysis and progressive severe muscle weakness with predominantly distal lower limb muscle involvement (1). SMARD1 is caused by recessive mutations in the gene encoding the immunoglobulin µ-binding protein 2 (IGHMBP2) on chromosome 11q13 (2). To date, IGHMBP2 cellular function and its role in the pathogenesis of motor neuron disease are not well understood.

IGHMBP2 is a member of the DEXDc DEAD-like superfamily of DNA/RNA helicases and is considered to act as a transcriptional activator and to be associated with pre-mRNA splicing complexes (3, 4, 5). Recent progress in the understanding of the pathogenetic mechanism of Spinal Muscular Atrophy (SMA) and other forms of motor neuron disease has revealed that lower motor neurons appear to have a specific vulnerability to defects in RNA metabolism (6). However, the subcellular distribution of IGHMBP2 not only in the nucleus but also, predominantly, in the cytoplasm and axons, suggests a possibly additional different cellular function in motor neurons (7). The neuromuscular degeneration mutant mouse (nmd) carries an autosomal recessive splice site donor mutation, reducing functional IGHMBP2 gene expression to 20–25%. These animals present with motor neuron degeneration which causes skeletal muscle fiber atrophy, progressive paralysis and premature death. As a model for human SMARD1, the nmd mouse provides a novel experimental tool to test new therapeutic strategies (8).

Motor neurons derived from Embryonic Stem Cells (ES) or from the activation of endogenous spinal cord stem cells represent a potential therapeutic strategy to reconstitute motor units in motor neuron diseases.

The differentiation of ES cells into motor neurons (MNs) has recently been reported by exposing ES cells - of both mouse and human origin - to retinoic acid (RA) and Sonic hedgehog (Shh) (9,10).
Motoneuron-committed ES cells transplanted into the spinal cords of adult rats with viral induced motoneuron injury survive and generate motor neurons in the anterior horns with a 25% differentiative efficiency (11).

Endogenous neural stem cells (NSCs) in the spinal cord capable of generating motor neurons can be obtained only during early development (E 10.5 in mouse) when the motor neuron genesis occurs. At this stage Neuroepithelial (NEP) multipotent stem cells can be isolated from the neural tube. At a subsequent stage the spinal cord contains neuronal/glial restricted progenitors while the number of NEP decreases to a negligible amount (12). In late embryonic development and during adulthood, spinal cord neurospheres could be obtained after FGF exposure in vitro. Previous studies reported that transplantation of spinal cord derived neurospheres into the injured spinal cord improves functional motor recovery (13, 14).

However, grafted NSCs, that did not receive an in vitro differentiation before transplantation, give rise in vivo preferentially to astrocytes, relatively few oligodendrocytes and rare neurons (13, 14, 15). These findings suggest that the functional improvement observed may be due to an indirect environmental effect, like the trophic support provided by astrocytes (16) and the remyelination of axons by donor derived oligodendrocytes (17).

Furthermore the capacity of spinal cord derived neurospheres to generate motor neurons is extremely limited also when tested in vitro (18). Identification of NEP in late embryonic development or in adult mammals may be useful to study motor neuron development and harness the potential of endogenous precursors as a source of new neurons for spinal cord repair.

Detection of Aldheide Dehydrogenase (ALDH) activity has been proposed as a method to isolate hematopoietic stem cells (19), and several groups have reported that high ALDH activity correlates with the stem cell state (20).

In this study we describe the isolation of ALDH High (ALDH$_{hi}$) Side Scatter low (SSC$_{lo}$) NSCs from embryonic and adult spinal cord. We show by clonal analysis that individual stem cells are self-renewing and multipotent. They are also able to generate motor neurons, in vitro and in vivo.
Furthermore, we investigated whether ALDH$^{hi}$SSC$^{lo}$ spinal cord cells could restore function to nmd mice. Transplantation of ALDH$^{hi}$SSC$^{lo}$ stem cells intrathecally in neonatal nmd mice resulted in partial restoration of function, whereas untransplanted nmd became paralyzed. The recovery of function was likely due to the reduction of host motor neurons death as well as to exogenous and endogenous neurogenesis.

Furthermore, to define the molecular events related to nmd motor neuron degeneration and identify the mechanism responsible for neuromuscular improvement in transplanted mice, we compared the lumbar spinal cord expression profile of transplanted and untransplanted nmd mice with the lumbar spinal cord of wild-type sib controls.
RESULTS

Isolation of ALDH expressing neural stem cells from spinal cord

Since high ALDH activity may correlate with the stem cell state, we utilized a fluorescent substrate (Bodipy®-aminoacetaldehyde BAAA termed Aldefluor) to monitor ALDH activity by Fluorescence Activated Cell Sorting (FACS) in foetal (E13.5) and adult spinal cord and neurospheres. We isolated a population of cells with a low side scatter (SSClo) and a high ALDH (ALDHhi) activity. ALDHhi and ALDHlo populations were re-analyzed after sorting and demonstrated more than 95% and more than 99% of purity, respectively (Fig 1 A-B).

ALDHhiSSClo cells represent 0.2-0.4% of dissociated adult tissues and 0.8-1.2% of foetal tissue as evaluated by FACS. ALDHhiSSClo cells are 3.8 ± 1.2% of embryonic neurospheres and 1.8 ± 0.7% of adult neurospheres (Tab 1).

These sorted populations (positive and negative fractions) along with unsorted cells were assayed for in vitro analysis. Cells were plated in growth NEP medium. ALDHhiSSClo cells appear as homogenous adherent cells that divide in culture and can be expanded at least for five passages.

To characterize their phenotype, after growth in culture for a period of 7 days, ALDHhiSSClo cells were tested for differentiation using a variety of antigenic markers. ALDHhiSSClo cells in basal condition expressed nestin (Fig. 1C) but did not express any neuronal or glial marker characteristic of differentiated cells. Furthermore these cells display expression features of neural and other stem cells, including Sox2 (Fig. 1 D) and musashi-1 (Fig. 1 E).

To confirm their self-renewing and multipotential properties, ALDHhiSSClo cells were grown at clonal density. ALDHhiSSClo cells from both embryonic and adult source generated primary colonies. The frequency of clone generating cells in the ALDHhiSSClo fraction from embryonic and adult spheres was 1 : 8 and 1 : 12 respectively. 1/17 cells deriving from dissociated embryonic tissue and 1/27 cells from adult tissue generate spheres (Tab. 1).
After 10 days of culture, the clones contained hundred to thousand of cells. When collected and replated at single cell level, these clones can generate secondary colonies.

FACS analysis demonstrated the presence of ALDH$^{hi}$SSC$^{lo}$ cells in secondary colonies ($6.6 \pm 2.4\%$ of secondary colonies of ALDH$^{hi}$SSC$^{lo}$ from embryonic spheres; $3.4 \pm 2.3\%$ from adult spheres (Tab. 1), confirming that the original ALDH positive fractions were self-renewing.

In the negative fraction, only a minor proportion generates cell clones predominantly sphere-like aggregates and rarely epithelial-like clones (Tab 1). In negative-derived secondary colonies the ALDH$^{hi}$SSC$^{lo}$ cells were less than $0.01\%$ of all cells.

Moreover, to determine whether ALDH$^{hi}$SSC$^{lo}$ cells could differentiate into mature spinal cord neurons, cells were grown in differentiation-promoting medium. Under these conditions, ALDH$^{hi}$SSC$^{lo}$ cells differentiated in the three major lineages, as confirmed by the morphology and the expression of lineage specific antigenic proteins in immunohistochemistry. We observed cells with tiny elongated processes expressing β III tubulin (TuJ1) (Fig. 1 G-I), neurofilament (NF) and microtubule associated protein 2 (MAP2) (Fig. 2 C), likely representing neurons. These cells increased with time and represented $42.6 \pm 4.4\%$ (embryonic) and $35.3 \pm 5.6\%$ (adult) of the total cell number. $15.2 \pm 4.2\%$ (embryonic) and $22.2 \pm 6.5\%$ (adult) cells were Glial Fibrillary Acid Protein (GFAP) positive. $5.3 \pm 2.2$ and $3.2 \pm 1.3\%$ of cells (adult and embryonic, respectively) expressed O4 antigen, indicating an oligodendroglial phenotype.

ALDH$^{hi}$SSC$^{lo}$ cells were also derived from Thy1-Yellow Fluorescence Protein (YFP) transgenic mice that express Green Fluorescence Protein (GFP) in all spinal cord neurons. To enhance the survival and differentiation of ALDH$^{hi}$SSC$^{lo}$ cells into motor neurons, we modified a previously published protocol by exposing them to Shh, RA, Nerve Growth Factor (NGF) and c-AMP. Under these conditions, cells presenting a larger cell soma and elaborate neuritic extensions were observed. These cells were positive for choline acetyltransferase (ChAT) (Fig 2 D-I), Islet-1 and HB9 that are motor neuron specific antigens (Fig. 1 J-L). We observed $27.6 \pm 6.6\%$ and $7.4 \pm 2.2\%$. HB9 positive neurons in embryonic and adult cells respectively.
To determine the potential of motor neuron-committed ALDH\textsuperscript{hi}SSC\textsuperscript{lo} cells to form neuromuscular junctions we established a co-culture with skeletal muscle-differentiated C2C12 myoblasts. ALDH\textsuperscript{hi}SSC\textsuperscript{lo}-derived neurons extended long axons with contacts with myotubes forming neuromuscular junctions, as demonstrated by bungarotoxin (BTX) staining that identifies clustered acetylcholine receptors. Confocal analysis demonstrated the interaction of YFP axons with BTX-acetylcholine receptors (Fig. 2 J-L).

**Transplantation of nmd mice with ALDH\textsuperscript{hi}SSC\textsuperscript{lo} stem cells**

We transplanted 20,000 ALDH\textsuperscript{hi}SSC\textsuperscript{lo} positive embryonic cells into the spinal fluid of nmd mice to investigate whether ALDH\textsuperscript{hi}SSC\textsuperscript{lo} cells have the capacity to differentiate appropriately within the spinal cord and modify disease progression. To trace the fate of transplanted cells we used ALDH\textsuperscript{hi}SSC\textsuperscript{lo} cells deriving from Thy1-YFP transgenic mice that express the gene reporter only in neurons. Moreover, we favored the use of embryonic cells for their greater replicative capacity and for their robust motor neuronal differentiation.

Animals were sacrificed at the end stage of the disease and were examined for the presence of YFP-expressing cells. We detected YFP\textsuperscript{+} cells both within the cervical and lumbar enlargement, indicating a pronounced migration of the transplanted cells. In addition, YFP cells were found both adherent to the meninges and within the spinal cord parenchyma.

YFP positive ALDH\textsuperscript{hi}SSC\textsuperscript{lo}-derived cells extended long processes often parallel to the longitudinal spinal tracts directed in the rostral-caudal projection. Some cells also extended processes horizontally in the gray matter, while only few cells extended in the white matter toward the ventral roots.

By carrying out an unbiased stereological quantification with optical disectors and random sampling, we estimated that a mean of 5,215 ± 73 YFP\textsuperscript{+} cells was present per spinal cord (mean 20.8 ± 6.6 YFP cells per section).
Since the primary method for detecting transplanted cells is the YFP expression that is limited to neurons, we performed FISH analysis for Y chromosome in sex mismatched transplantation experiments (male cells into female recipients) to evaluate the number of total donor derived cells present. Based on this analysis, we predicted that 12,967 ± 1120 male donor cells were present in the spinal cord parenchima. To determine the ability of ALDH^hiSSC^lo cells to acquire neural cell phenotype in vivo, we performed immunohistochemical analysis for neuronal markers followed by confocal microscopy on the spinal cord sections of transplanted animals. ALDH^hiSSC^lo YFP cells located in the spinal cord gray matter were immunoreactive for several neuronal specific markers, including TuJ1, MAP2, and Nuclear Neural-specific Antigen (NeuN).

Immunohistochemical analysis for glial antigens (GFAP and O4) combined with FISH analysis for Y chromosome revealed the presence of 28.4 ± 4.6% astrocytes and 3.6 ± 1.2 % oligodendrocytes of all donor male cells. MAP positive Y positive mature neurons were 39.8 ± 4.5% of all male cells, while cells expressing the more immature antigen TuJ1 were 46.6 ± 4.7%. Furthermore 19.6 ± 5.6 % of cells expressed nestin.

Moreover, we tested whether transplanted cells display a motor neuronal phenotype by evaluating the expression of choline acetyltransferase (ChAT) and HB9. These cells morphologically resembled true motor neurons and are quantified to almost 20.5 ± 6.3% of all YFP neurons (1069.07 ± 328.54 YFP positive motor neurons per spinal cord) (Fig. 3).

To investigate whether engrafted ALDH^hiSSC^lo cells extend processes from the ventral horn into the periphery, we examined ventral roots of transplanted animals and observed a mean of 35 ± 15 YFP positive axons per animal (5.1 ± 2.1 % of all axons in the anterior root). To evaluate ALDH^hiSSC^lo cells migration at short time, dye fluorescent (PKH26) labelled cells were delivered into the CSF. 1-2 days after cell injection, a large number of donor cells (87.7 ± 5.6 % of injected cells) were distributed extensively on the surface of the spinal cord. One week after transplantation, grafted cells survived and proliferate generating cell clusters attached to the pia mater of the spinal cord. Few cells were observed to migrate into the spinal cord through pia mater. Cells invasion was observed also at root exits. Cell clusters were situated preferentially near the blood vessels probably
favoured by the nutrient supply. This location may also allow parenchymal distribution. In the brain of recipient animals, few cells were detected, particularly in the area directly adjacent to the ventricular system. The cell attachment on the spinal cord surface as well as the cell cluster formations were less frequent in non mutated wild-type mice. Spinal cord parenchymal cell invasion was extremely rare.

**Comparative analysis of clinical symptom development and survival between nmd transplanted and untransplanted mice**

*Nmd* mice present the first clinical symptoms at the second postnatal week. Subsequently, they rapidly develop muscle weakness starting in the hindlimbs that are dorsally contracted. Their locomotor activity is impaired, but balance is not adversely affected. The limited limb extension determines the impossibility to stand on all four limbs. Homozygotes clench their hindlimbs when picked up by the tail and are unable to grasp a cage cover when held against it. Later the weakness is generalized to involve the forelimbs as the disease progresses.

At three weeks, in *nmd* treated mice the hindlimb muscles were almost normal in contrast to their *nmd* littermates who presented muscle wasting and marked contracture of the hind limbs (Fig. 4 D). Mutant mice were severely paralyzed at the age of 5 weeks. At the age of 5 weeks, all *nmd* mice failed on the rotarod test and could not stay on the accelerating wheel for longer than few seconds (*p* < 0.0001 vs. control sibs).

In contrast, transplanted *nmd* mice at 3 weeks of age did not present typical hind limb posture and at 5 weeks of age they were still able to perform rotarod test (*p* < 0.0001 vs. untreated), even if they presented a moderate decrease in performance when compared to their wild-type sibs. At the successive time point treated *nmd* gradually lose their ability to execute rotarod test, but with performance significantly superior to the untreated animals (*p* < 0.0001 vs untreated *nmd* mice until 8 weeks).
Growth rates for transplanted and untransplanted *nmd* mice, as measured by mean body weight, were almost indistinguishable from their control littermates from birth up to 1 week of age. However, during the second and third week of life, *nmd* mice present a significantly reduced mean body weight if compared with their control sibs (21 days, 4.4 ± 0.44 g, n= 24 versus 9.4 ± 0.41 g, n=24, \( p < 0.0001 \)). Transplanted *nmd* mice displayed an intermediate growth rate significantly different from untreated *nmd* mice and from controls (7.1 ± 0.86 g, n= 24, \( p < 0.0001 \)) (Fig. 4 A).

Both male and female *nmd* transplanted mice displayed significantly increased mean life spans than their gender-matched and sib-matched *nmd* mice (treated males (n = 12): 74.9 ± 13.3; treated females (n = 12): 88.3 ± 17.1 vs. untreated males (n = 12): 56.2 ± 13.1; untreated females (n = 12): 70.2 ± 12.7 \( p < 0.0001 \)). Gender differences in lifespan of *nmd* mice and other motor neuron disease animal models have been previously described (21, 22).

Additionally, the maximum life span (107 for treated males and 127 days for treated females) was higher than those of untreated *nmd* mice (76 for treated males and 99 days for untreated females) (Fig. 4 C).

**Comparative analysis of motor neuron and axon loss in transplanted and untransplanted *nmd* mice**

To investigate the reasons for the motor and survival improvement in *nmd* transplanted mice we studied spinal cord motor neurons and ventral spinal nerve roots from a quantitative point of view. Compared to wild-type littermates, a severe loss of spinal motor neurons was observed prior to the onset of clinical symptoms at 2-3 weeks, while at the age of 6 weeks motor neuron loss was reduced to 40%. The cytoplasm of degenerating spinal motor neurons in *nmd* mice was characterized by severely reduced Nissl substance, which was particularly apparent in 6-week-old mice, while the nucleolus diameter was preserved (Fig. 5 A-F).
At 6 weeks, we observed a substantial reduction of motor neuron loss in the lumbar spinal cord of transplanted mice with 63% of motor neurons in comparison to non-transplanted mice ($p < 0.0001$) (Fig. 5 J).

At the final end stage of the disease the number of motor neurons of untreated animals was reduced to 24% while transplanted animals conserved 40% of motor neurons (treated vs untreated nmd $p < 0.0001$) (Fig. L). Significant neuronal loss was evident in 6-week-old nmd mice, as shown by a significant reduction in the diameter of L4-L5 ventral nerve root cross-sections. Similar reductions were observed in all lumbar ventral roots examined. In contrast, a modest decrease was observed in the diameter of ventral roots of age-matched transplanted mice. Nmd ventral root sections revealed a reduction in large caliber fibers with dystrophic features and a relative increase in small caliber axons (Fig. 5 G-I). The transplanted animals presented a partial preservation of large axon density. The quantitative assessment revealed that more than half (55%) of L4 motor axons were lost in untransplanted mice ($595 \pm 25$ vs. $1082 \pm 24$ wild type B6 animals, $p < 0.0001$) while in transplanted nmd mice this loss was significantly reduced, preserving 75% of the control number of axons in L4 ventral nerve roots ($810 \pm 29$ vs. untreated, $p < 0.0001$).

In particular, we observed a major loss in large axons ($119 \pm 9$ versus $488 \pm 11$ axons in B6 controls, $p < 0.0001$), followed by small ($220 \pm 12$ versus $314 \pm 7$ axons, $p < 0.0001$) and medium ($256 \pm 12$ versus $280 \pm 7$ axons, $p < 0.0023$) caliber myelinated axons in untreated animals. On the other hand, we detected an intermediate loss of axons in transplanted nmd mice with a less evident reduction in large ($243 \pm 9$ axons vs. untreated, $p < 0.0001$), small ($307 \pm 11$ axons, $p < 0.0001$) and medium ($260 \pm 9$ $p = 0.4556$) caliber axons compared to untreated mice (Fig. 5 K).

At disease end-stage, the quantitative evaluation of L4 anterior roots showed a severe loss in large axons in untransplanted mice ($72 \pm 7$ versus $486 \pm 11$ wild type B6 animals, $p < 0.0001$), while in transplanted mice this loss was significantly lower ($152 \pm 10$ vs untreated, $p < 0.0001$) (Fig. 5 M).

**Microarray and Real Time PCR Analysis**
To identify the mechanism responsible for neuromuscular improvement in transplanted mice and define the molecular events related to \textit{nmd} motor neuron degeneration, we compared the lumbar spinal cord expression profile of transplanted and untransplanted \textit{nmd} mice with wild-type sib controls (at 6 weeks of age) by Microarray and Real-Time RT-PCR analysis.

Differences in the gene expression level are presented as ratios of the mean values between untransplanted \textit{nmd} mice and wild-type, transplanted \textit{nmd} mice and wild-type, transplanted \textit{nmd} mice and untransplanted \textit{nmd} mice.

Setting a significant level of 2.0-change and excluding genes with low expression level, we detected 138 genes which varied their expression levels in the spinal cords of mutant transplanted, untransplanted \textit{nmd} and control mice. The hierarchical cluster analyses clearly discriminated the expression profiles of the three experimental conditions (Supplementary online Fig. 1).

The genes predominantly altered in \textit{nmd} untreated mice compared to wild-type mice are listed in Tab 2 A, B. An equal number of genes were down-regulated (n: 17) and up-regulated (n: 17) in the spinal cords of the 6-week-old \textit{nmd} mice, when compared with those of their littermates.

A large body of evidence suggests that two pathogenic mechanisms are likely to be involved in motor neuron degeneration: the excitatory amino acid (EAA) toxicity and oxidative stress. Both these pathways appear to induce neuronal damage by increasing intracellular Ca$^{2+}$ levels (23).

We observed a reduction of genes related to calcium ion binding, like parvalbumin and EF hand domain containing 1, in \textit{nmd} mice. Since one of the mechanisms used by cells to control increased intracellular Ca$^{2+}$ is through binding of this element to several cytosolic calcium binding proteins (CBPs), the reduction of CBPs in spinal cord may increase the motor neurons susceptibility to these pathogenic mechanisms (24). However the observed reduction in calcium binding protein might not be motor neuron specific, since parvalbumin is expressed at very low levels in the motor neurons even in normal mice (25).

It has been suggested that in the spinal cord the extracellular glutamate concentration has to be kept low enough to terminate glutamate receptor activation and protect neurons from glutamate excitotoxicity on motor neurons (23). In mutated and treated mice, we observed an increase in the
glial high affinity glutamate transporter (GLAST) while a reduction in another solute carrier, the solute carrier family 9 (sodium/hydrogen exchanger) isoform 3 regulator 2 (Slc1a3) was detected. Among the down-regulated genes we observed a decrease in adenylate cyclase-associated protein 1 (Cap1)-encoding mRNA. Cap1 contributes to dynamic remodeling of the actin cytoskeleton and plays an important role in cell morphogenesis, motility, and receptor-mediated endocytosis (26). Phosphodiesterase 8A that hydrolyzes cAMP with high affinity was reduced as well. Moreover, a reduction was also noted in various proteins that are integral to membrane such as tumor necrosis factor alpha induced protein 6 (Tnfaip6), 2410018G23Rik, myelin and lymphocyte protein T-cell differentiation protein (Mal), and secreted phosphoprotein 1 (Spp1).

The up-regulated genes include the ion channel protein FXYD6 and some genes related to the structural organization of DNA into chromatin such as DNA methyltransferase 3 A (Dnmt3a) and histone 1 transcripts that play a role in epigenetic control of gene expression (27).

We also observed an increase in the angiotensin receptor-like (Apelin receptor) in mutated mice, while this transcript was reduced in treated mice. Apelin is a G protein-coupled receptor expressed on neurons probably acting as neuromodulator (28).

In both mutant and treated mice, there was an up-regulation in the Igfbp5 gene that plays an important role during Central Nervous System (CNS) development in association with Insulin-like Growth Factors, IGF I and II (29, 30).

To verify the validity of the gene expression levels detected by microarray analysis, we performed quantitative Real-Time RT-PCR analysis on some genes of interest which demonstrated mean fold changes in expression levels directionally similar to those determined by microarray analysis. The increased or decreased gene expression levels were expressed as the ratio of the genes of interest compared to the ones expressed in wild type mice. Cap1 and Parvalbumin were confirmed to be significantly decreased in mutated mice (untreated mice: Cap 1: 0.45 \(p < 0.01\); Parvalbumin: 0.47 \(p < 0.01\)). Among the upregulated genes, FXYD6 was increased 3.12-fold \((p < 0.01)\) in untreated \textit{nmD} and 4.65-fold in treated \textit{nmD}, DNMT3a was augmented 2.1-fold \((p < 0.01)\) in treated and 2.0 fold in untreated mutated mice, while Slc1a3 was increased 2.6 in untreated mice and 3.0 in
treated ones ($p < 0.01$). The Igfbp5 gene was increased 2.1-fold ($p < 0.01$) in untreated mice and 9.58-fold ($p < 0.01$) in transplanted mice.

Transplantation of ALDH cells leads to de novo neurogenesis

Microarray gene expression analysis revealed a remarkable difference in gene expression profile between nmd treated and untreated mice (Tab 3 A, B).

Interestingly, we noted that the majority of the upregulated genes expressed in transplanted mice are involved in neurogenesis processes.

In particular, a group of cytoskeletal proteins, which are peculiar to the CNS development, seem to be overexpressed in transplanted mice compared to non-treated ones. This group includes Doublecortin (DCX), the microtubule-associated protein tau, the platelet-activating factor acetylhydrolase-isoform 1b-alpha1 subunit that interacts with LIS1 microtubular associated protein and drebrin 1 that has been known to act on actin filament at dendritic spines of neurons and motor neurons. Among early postmitotic neuronal markers we observed a four-fold increase in the expression of the dihydropyrimidinase-like 3 and CD24a. We also observed a nine-fold increase in the sialyltransferase 8 gene that is responsible for the synthesis of b-series gangliosides and triggers cholinergic neuritogenesis as well as neurite outgrowth and differentiation. Also, genes associated to neuronal transcription factor activity, like differentiation 6 ectodermal-neural cortex 1, homeobox B5 and development retinol binding protein 1 were up-regulated in treated mice.

We hypothesized that the increased expression of proteins related to neurogenesis may be attributed not only to the newly transplanted neurons but also to an increased endogenous neurogenesis. DCX is a microtubule-associated protein that is expressed specifically in virtually all migrating neuronal precursors of the developing CNS and has recently been proposed as an accurate measurement of the adult neurogenesis rate (31).

We therefore investigated the DCX expression pattern within the treated nmd spinal cord (Fig. 6). The target was to characterize the cellular identity of DCX-expressing cells, testing their
endogenous or exogenous origin. In 43.7 ± 7.7% of all YFP neurons confocal analysis revealed a co-localization with DCX signal. However, a fraction of doublecortin-positive neurons failed to co-localize with YFP and possibly constituted a distinct population of newly generated endogenous neurons.

These cells represent both immature neurons still not expressing YFP and endogenous new neurons. Immunohistochemical analysis for DCX associated with FISH for Y chromosome in sex mismatched transplantation (female donor and male recipient) demonstrated the presence of 887 ± 56 DCX+Y+ newly generated endogenous neurons in nmd treated spinal cord.

No expression of DCX was detected in the untreated and wild-type spinal cord. The expression of doublecortin in treated nmd spinal cord was confirmed also by Western blot (Fig. 6 G). This analysis showed two doublecortin bands, demonstrating the expression of both unphosphorylated and phosphorylated forms (32).
DISCUSSION

In the present study we demonstrate the ability of YFP positive ALDH<sup>hi</sup>SSC<sup>lo</sup> stem cell derivatives to engraft diffusely throughout the spinal cord, to generate motor neurons and improve the phenotype and survival of nmd mice, an animal model of SMARD1.

The aim of our experiment was to isolate a primitive population of spinal cord stem cells with the property to differentiate in motor neurons. At present, only ES and NEP seem to own the capacity to differentiate in motor neurons while only rare cholinergic cells are obtained from spinal cord neurospheres. Stem cells from brain and spinal cord deriving from both adult and embryonic stages are usually isolated retrospectively by their ability to form neurospheres in vitro. However, neurospheres are not a pure homogenous cell population and contain only a small stem cell fraction. Moreover the absence of specific markers for NSCs brought up difficulties concerning their identification and isolation.

ALDH activity has been used to isolate hematopoietic progenitors (19) and has been proposed as a common stem cell marker for cells derived from different tissues, in association with other properties such as ABCG2 and telomerase activity (20). A possible emerging hypothesis regarding the origin of these shared characteristics is the existence of a “stem cell state” partially determined by the niche in which stem cells reside (20). Rat embryonic multipotent neural stem cells from E10.5 spinal cord also express high level of ALDH activity, but this property was not previously used to enrich NSC cells (20).

In this study, we isolated NSCs from embryonic and adult spinal cord, based on the high level of ALDH expression. These cells are self-renewing and multipotent. They are characterized by the expression of nestin and can be maintained as adherent cells in culture at clonal density. ALDH<sup>hi</sup>SSC<sup>lo</sup> cells appear flattened, epithelioid and grow in a tightly dense monolayer. Furthermore, they require Fibroblast Growth Factor (FGF) to proliferate and maintain an undifferentiated phenotype in culture. Under appropriate environmental conditions ALDH<sup>hi</sup>SSC<sup>lo</sup>
cells differentiate into the three principal lineages and also acquire mature complex neuronal phenotypes including that of HB9/ChAT-expressing motor neurons. For these characteristics we hypothesized that ALDH\(^{hi}\)SSC\(^{lo}\) cells could represent a homogenous population of NEP-like cells that persist in later embryonic and adult life.

On the basis of these observations, we also investigated whether ALDH\(^{hi}\)SSC\(^{lo}\) cells can generate motor neurons in vivo in \textit{nmd} mice.

Since the motor neuron disease involves the entire spinal cord, we adopted a CSF-based delivery system that distributes cells in a uniform and widespread way and represents a minimally invasive route of administration.

Previous studies have demonstrated that transplantation of NSCs promotes functional recovery in injured spinal cord. Most of these studies were based on direct injection of cells into parenchymal lesion (13, 14, 15). This is an effective strategy to efficiently deliver cells into the injured areas, however in the perspective of a clinical application this technique presents several practical issues including major invasive surgical procedures along with an additional risk of damage of the spinal cord. Furthermore most of spinal cord diseases, such as motor neurons degeneration are diffuse requiring transplantation over the entire spinal cord length. The development of CSF delivery method could permit a widespread, minimally invasive administration of the cells.

NSCs can also be delivered via intraventricular and intratechal injections in the injured CNS. Transplanted cells migrate toward the disease sites, proliferate and differentiate also in neuroglial mature phenotypes (33, 34, 35).

In this study, we report that ALDH\(^{hi}\)SSC\(^{lo}\) cells can efficiently reach the spinal cord of \textit{nmd} mouse model via intrathecal delivery.

Transplanted ALDH\(^{hi}\)SSC\(^{lo}\) cells were disseminated over the entire rostrocaudal length of the spinal cord and were engrafted over a substantial spinal cord area. Furthermore, we demonstrated that ALDH\(^{hi}\)SSC\(^{lo}\) cells can migrate across the meningeal layer into the spinal cord. The ability of NSCs to survive in the CSF and to migrate through the pia mater is a critical requisite in the successful engraftment of these cells. In addition to homing ability, appropriate neuronal differentiative
potential allows functional donor cell integration into host tissue. Indeed, we found that the transplanted NSC engrafted the diseased spinal cord, but only minimally engrafted the intact spinal cord of wild-type nmd littermates, suggesting that chemotactic signalling and adhesion molecules of host degenerated tissues are likely to play a significant role by recruiting NSCs. Furthermore, motor neuron cell death can open new niches that can be occupied by the donor cells.

Our results demonstrate that ALDH^{hi}SSC^{lo} cells may differentiate within the spinal cord into neurons and motor neurons after transplantation, survive in the gray matter, migrate, extend processes, and acquire distinct neurotransmitter profiles. Moreover, the newly formed neurons appear well integrated into the host environment and persist up to 2-3 months in vivo.

The adult mammalian spinal cord is a non-neurogenic site, likely due to a non-permissive environment for the generation of new neurons. We committed ALDH^{hi}SSC^{lo} cells to a motor neuron fate before transplantation and found the presence of ALDH^{hi}SSC^{lo} cell-derived motor neurons within the spinal cord of transplanted mice. The efficient generation of new neurons in non-neurogenic regions of the neuraxis and their extensive survival within the adult mammalian spinal cord could be obtained through appropriate ex vivo pre-treatment.

To exert a normal functional activity, donor motor neurons must successfully connect with their muscle targets. Several groups have described the generation of cholinergic neurons from mouse ES cells and human cells in vitro by treating them with inductive factors such as retinoic acid and sonic hedgehog (9, 10).

Actually, ES cell-derived motoneurons form functional neuromuscular junctions in vitro. However when transplanted into spinal cords of adult rats with motoneuron injury they fail to send their axons to the periphery. ES cell-derived axonal growth seems inhibited by myelin that can be partially overcome by administration of dibutyryl cAMP. In these conditions ES cell-derived motor axons were observed within the ventral roots of each animal, but not in the neuromuscular junctions. Furthermore functional improvement was not described (11).

In our experiments, ALDH^{hi}SSC^{lo}-derived motor neurons have the ability to generate in vitro neuromuscular junctions when co-cultured with myotubes. In vivo, we observed that donor-derived
motor neurons extend axons through the CNS white matter and in the ventral roots. This can be due to the beneficial effect of c-AMP pre-treatment or to the more permissive environment for axon extensions of a neonatal spinal cord compared to adult spinal cord.

The axons’ limited regeneration is probably due to myelin-associated inhibitors of axonal growth such as myelin-associated glycoprotein, Nogo-A, and oligodendrocyte-myelin glycoprotein. All of these three proteins bind to the Nogo-66 receptor and are likely to transmit a signal through the P75 neurotrophin receptor (36). Signaling via this pathway may decrease intraneuronal cAMP and differentially activate members of the Rho GTPase family (37). However, according to recent reports, human neural stem cell-derived motor neurons - transplanted into rat with neonatal sciatic axotomy - may send axons through the ventral root and sciatic nerve to form neuromuscular junctions with their muscle targets (38).

These different findings may be due to different injury models, acute vs. chronic injury, differences in cell type, and different treatment of stem cells prior to grafting. Modulation of these factors may hopefully lead to a degree of muscle innervation efficient enough to produce a clinical therapeutic effect. Transplanted nmd mice showed an amelioration of disease as demonstrated by growth curve and neuromuscular function test (rotarod) and an increased survival. We hypothesized that this beneficial effect was due to a chain of events induced by stem cells transplantation: the exogenous and endogenous neurogenesis and the protective environmental change on endogenous motor neurons. Astrocytes are part of this microenvironment and they are known to play a complex role in determining neuron survival in motor neuron diseases, such as Amyotrophic Lateral Sclerosis (ALS), also through trophic support and glutamate transport (39). In fact, we observed the genesis of donor derived astrocytes, demonstrated by the double staining for the Y male chromosome and GFAP, in the recipient’s female spinal cord. Furthermore, the microarray profile analysis demonstrated an increased GLAST expression in transplanted nmd mice. GLAST is a specific astrocyte marker linked to a functional role of astrocytes in glutamate homeostasis (40).

Motor neuron and axon count in treated compared to untreated mice, demonstrated a significant reduction of neural cell death which represents the most relevant result in terms of clinical benefit.
Indeed, the number of donor derived motor neurons extending their axons through the ventral root toward the periphery is too limited to account for the functional recovery itself. Still, the demonstration that motor neurons can be generated from ALDH\textsuperscript{hi}SSC\textsuperscript{lo} cells after transplantation with a minimal invasive CSF injection, points out a new possible approach for the replacement of dead motor neurons. Furthermore, the presence of \textit{de novo} neurogenesis may play a significant role in the observed clinical improvement.

Several evidences suggest that neurogenesis per se may contribute in a significant way to neural repair. In fact, a functional neurological improvement has been associated to increased neurogenesis, induced either by stem cells or growth factors administration (41, 42). This positive correlation has been observed not only for brain neurogenesis but also after grafting of ES and marrow stromal cells, as well as NSCs, into the injured spinal cord (43, 44, 45). The precise mechanisms that correlate neurogenesis to repair of function have not been clearly determined yet.

To investigate the molecular events responsible for phenotypic differences observed after cell transplantation, microarray and Real time RT-PCR analysis of wild-type, mutated and transplanted \textit{nmd} spinal cord were performed. We isolated a number of transcripts with a significant differential expression in the lumbar spinal cord of mutant transplanted and untransplanted mice compared with littermate controls. At 6 weeks (i.e. when gene expression was studied) \textit{nmd} mutant mice displayed manifest signs of disease, while transplanted mice presented an intermediate phenotype between normal and mutated mice. We observed an alteration of genes known to be involved in motor neuron degeneration like calcium metabolism and glutamate uptake. In particular, \textit{nmd} mice showed a reduction of parvalbumin transcript, which codes for a calcium ion binding protein of lower motor neurons. Calcium-dependent death is considered one of the pathogenetic events responsible for motor neuron degeneration. Consistently, over-expression of parvalbumin in transgenic mice rescues motor neurons from injury-induced cell death (25). Therefore, the reduction of CBP expression may render motor neurons vulnerable to the increased intracellular Ca\textsubscript{2+}, even if parvalbumine’s expression might not be motor neuron specific.
Moreover, we observed an increase in the glial high affinity glutamate transporter (GLAST) and a reduction of the solute carrier family 9 (sodium/hydrogen exchanger) isoform 3 regulator 2. An increase in the expression levels of GLAST in human motor neuron disease tissues has been reported (46). Glutamate excitotoxicity is implicated in the etiology of motor neuron diseases with impairment of glutamate transport as possible cause of glutamate-induced injury to motor neurons. A reduction in the astrocyte glutamate transporter GLT-1 has been described in ALS patients (47) and in the spinal cord of SOD1 mice (48). The opposite expression changes of GLAST and GLT1 in our model indicate that the role of glutamate transporters in the pathogenesis of motor neuron disease might turn out to be more complex than previously appreciated. Yet, it should be noted that we observed an increase in GLAST expression in treated mice as well, although this might be partially attributed to the presence of the exogenous NSCs. In fact, glutamate plays critical roles in synaptic plasticity (49) and is highly expressed during spinal cord development (50). Furthermore, we detected an up-regulated expression of genes related to heterochromatin maintenance like Dnmt3a and histone 1 transcripts. Chromatin modification may be involved in the establishment and maintenance of cell memory in neurogenesis and in the production of motor neurons, during embryonic development (27).

In addition, we observed an up-regulation of the IGFBP5 gene in mutant and treated mice. The insulin-like growth factors, IGF-I and IGF-II, and their binding proteins (IGFBPs) are widely expressed and temporally and spatially distributed in the CNS, suggesting that they play an important role during brain development (29, 30). In neurogenesis, IGFBP-5 has been shown to co-localize with IGF-I or in the vicinity of IGF-I-producing neurons, which suggests that IGFBP-5 can modulate the action of the IGFs (51). The positive relationship between IGFBP-5 and IGF-I activity manifests itself in the increased IGFBP-5 expression in brain tissue overexpressing the IGF-I gene (52). IGF-I is a potent survival factor for motor neurons and it has been tested as a possible therapeutic agent for ALS. It has been verified that in the ventral horns of ALS patients, free IGF-I is reduced while IGFBPs 2, 5, and 6 are increased in the spinal motor neurons (53). The increased level of IGFBP5 in mutant mice may represent an endogenous attempt to increase the IGF
concentration in the offended zone in response to the motor neuron damage. The high level of IGFB5 expression in transplanted mice may reflect the embryonic origin of donor cells in view of the high IGFB5 production characteristic of developmental stage. IGFBP5 may play a positive role both in enhancing the IGF1 effect and maintaining its concentration level. Furthermore, IGFBP5 may act independently from IGF on neuron proliferation and differentiation.

The analysis of the detected up-regulated genes in treated mice gave rise to the interesting observation that many of these transcripts are related to neurogenic processes.

These findings are not only due to the presence of transplanted neurons but also to increased endogenous neurogenesis as demonstrated by the expression analysis.

Both exogenous and endogenous neurogenesis could potentially contribute to the observed phenotypic improvement. Transplantation of different types of adult somatic stem cells has been previously described to induce neurogenesis, but the molecular pathways involved in the induction of neurogenesis after stem cell transplantation remain unknown (41).

The data originated from microarray gene expression analysis demonstrated a significant upregulation of IGFBP5 that may therefore be in some way involved in this process. One possible hypothesis is that cell-to-cell direct interaction may contribute to the activation of these neurogenesis events. As it has been previously demonstrated cell-to-cell direct contact promotes astrocytes-induced neurogenesis (54).

Despite the fact that nmd transplanted mice display a remarkable amelioration of the neurogenic atrophy phenotype their exitus remains premature. The motor neuron and axon count at the end stage of the disease demonstrates that despite the progressive motor neuron loss in nmd treated mice, their number and the corresponding axon fibres, remain significantly higher than those observed in untreated animals. These observations support the hypothesis that premature death of transplanted animals is probably due to both an only partial rescue of the phenotype and to a multi-organ involvement. In particular heart failure might be responsible for nmd mouse death. The generation of transgenic nmd mice expressing the normal full-length IGHMBP2 cDNA only in neurons was recently described (21). These animals present normal motor neuronal features but die
prematurely due to heart failure. These findings suggest that IGHMBP2 deficiency in nmd mice compromises the integrity and function not only of motor neurons but also of skeletal and cardiac myocytes, suggesting that IGHMBP2 also plays a primary role in muscle tissue dysfunction. Probably complete functional rescue of the nmd phenotype will require improvement not only of motor neurons but also of cardiac and skeletal muscles (21).

We consider that further investigation on the stem cell biological properties in the spinal cord and their environmental interactions may harness their potential in mobilization of endogenous cells as possible therapeutic strategy for spinal cord regeneration.
MATERIALS AND METHODS

Isolation and culture of neural stem cells

Neural stem cells were isolated from the spinal cord at embryonic day 13.5 and from the adult spinal cord of C57BL/6 mice as described previously (55).

Isolated spinal cords were mechanically dissociated with Pasteur pipette and incubated in 0.05% trypsin/EDTA solution for 15 min at 37°C. The cell suspension was spun at 1000 rpm for 5 min, and re-suspended in culture medium.

Single cell suspension was seeded at a density of 100,000 cells/ml in Neurobasal medium (GIBCO™ Invitrogen, Carlsbad, CA), containing B-27, N2 (Invitrogen), Epidermal Growth Factor (EGF) (20 ng/ml, Sigma-Aldrich St. Louis, MO), bFGF (20 ng/ml, Sigma-Aldrich), penicillin (100 U)/streptomycin (100 µg/ml; Invitrogen). Cells were grown in uncoated T75 plastic flasks (NUNC, Nalgene Nunc International Corp., IL, USA) as free-floating clusters (neurospheres).

The cultures were passaged every 5-7 days. Cells used for separation had been passaged three to five times.

Selection of ALDH<sup>hi</sup>SSC<sup>lo</sup> cells

ALDH<sup>hi</sup>SSC<sup>lo</sup> cells were isolated from freshly dissociated spinal cord or from spinal cord neurospheres. For ALDH staining, the cells were suspended in Aldefluor assay buffer containing ALDH substrate, BAAA (1 µmol/l) for 60', following the manufacturer's instructions. After staining, cells were maintained in ice during all subsequent procedures. In each experiment a sample of cells was stained under identical conditions with 50 mmol/l of specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) to serve as negative control. Flow cytometric sorting was conducted using a FACS Vantage SE (Becton Dickinson Immunocytometry System, Mountain
Aldefluor fluorescence was excited at 488 nm and fluorescence emission was detected using a standard fluorescein isothiocyanate (FITC) 530/30 bandpass filter. Low side scatter (SSC<sup>lo</sup>) and high ALDH (ALDH<sup>hi</sup>) were selected.

**Cell culture and differentiation of ALDH<sup>hi</sup>SSC<sup>lo</sup> positive cells**

For cell expansion and clonal culture, ALDH<sup>hi</sup>SSC<sup>lo</sup> sorted cells were plated in a previously described growth medium (NEP medium), containing FGF with or without EGF (56). Differentiation of cultured ALDH<sup>hi</sup>SSC<sup>lo</sup> cells was induced by plating on poly-lysine/laminin-coated dishes (poly-lysine at 20 µg/ml in DPBS; Sigma) and reducing FGF concentration with the addition of RA (1 µM). Cell motor neuron differentiation was carried out as previously reported with slight modifications (9). ALDH<sup>hi</sup>SSC<sup>lo</sup> cells were exposed to RA/Shh and also to c-AMP and NGF. For co-culture with myoblasts, ALDH<sup>hi</sup>SSC<sup>lo</sup> cells priming into MN were seeded on C2C12 myoblasts (American Type Culture Collection) induced to differentiate in Muscle differentiation medium (Promocell, Heidelberg, Germany) for 2 days.

**Immunocytochemistry on cell culture**

Cultured cells were fixed in 4% paraformaldehyde (PFA, 10 min) at Room Temperature (RT). After rinsing with phosphate-buffered saline (PBS), and pre-incubation in a mixture of 5% normal serum and 0.25% Triton X-100 in PBS, the cultures were incubated with the primary antibodies (see below) overnight at 4°C. The following proteins were evaluated: nestin (mouse monoclonal, 1:200, Chemicon, Temecula, CA); vimentin (mouse monoclonal, 1:200; Chemicon), Sox2 (rabbit, 1: 200, Chemicon); Musashi (rabbit, 1: 200, Chemicon); TuJ-1 (mouse monoclonal, 1:200; Chemicon), NF-M and H phosphorylated (mouse monoclonal, 1:200; Chemicon), NeuN (mouse monoclonal, 1:100; Chemicon), mouse monoclonal anti-MAP2 (1:100 dilution; Sigma-Aldrich), rabbit anti-ChAT (1:100; Chemicon), rabbit anti-Insulin gene enhancer protein (Islet-1) (1:200; Chemicon), rabbit
anti-HB9 (1:200; Chemicon), mouse Cy3 conjugated GFAP (1:400 dilution; Sigma-Aldrich), anti-
O4 (mouse monoclonal, 1:100; Chemicon) and Alexa 488 rabbit polyclonal antibodies recognizing
GFP (1:400; Molecular Probes, Eugene, OR), rhodamine-conjugated bungarotoxin was purchased
from Molecular Probes (T-1175; 1:1,000).

After repeated rinses in PBS, the primary unconjugated antibodies were further incubated with
FITC and RPE or tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibodies
(1:100; DAKO, Carpinteria, CA) (1 h, dark, RT) in PBS, then rinsed in PBS and coverslipped.

Controls with omission of primary antibodies were made, with no detection of positive signals.

For quantitative analyses of cell phenotypes of ALDH$^{hi}$SSC$^{lo}$ differentiated in vitro, ten monolayer
fields (more than 200 cells) were randomly chosen for each sample. The percentage of any given
phenotype in a sample was obtained by averaging proportions of a specific cell type in each of the
10 fields. At least four samples were counted for each treatment group.

Mice

B6.BKS Ighmbp2$^{nmd-2J}$ mice heterozygous for the nmd2J mutation were intercrossed and
homozygous mutant mice and wild-type littermates were used for analyses. Mice were genotyped
as described (8).

As cell donor we used transgenic mice expressing fluorescent protein. B6.Cg-TgN(Thy1-YFP)16Jrs
mice express spectral variants of GFP (such as YFP) at high levels in motor and sensory neurons, as
well as in subsets of central neurons (57). Genotyping was performed by PCR as described (57).
The transgenic construct contains a YFP gene under the direction of regulatory elements derived
from the mouse Thy1 gene. These elements are composed of a 6.5 kb fragment obtained from the
50 portion of the Thy1 gene, extending from the promoter to the intron following exon 4. Exon 3
and its flanking introns are absent. The deleted sequences are required for expression in non-neural
cells but not in neurons. The remaining sequence is required for neuronal expression (57).
All transgenic animals were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed according to institutional guidelines that are in compliance with national (D.I. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circolare No.8, G.U., 14 Luglio 1994) and international law and policies (EEC Council Directive 86/609, OJ L358, 1 Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

**ALDH**\(^{hi}\) **SSC**\(^{lo}\) **Cell Transplantation**

**ALDH**\(^{hi}\) **SSC**\(^{lo}\) positive cells from Thy1-YFP embryonic spinal cord were used for transplantation after FACS selection. One- to 2-day-old **nmd** mice pups were used as graft recipients.

Cells were transplanted into the CSF of cryoanesthetized animals as previously described (58). 2 µl of cell suspension (20000 cells) were slowly injected.

To completely evaluate the fate of donor cells in addition to neuronal differentiation allowed by Thy1-YFP expression, sex mismatched transplantation was performed by transplanting male cells into female recipients and vice versa.

To easily evaluate cell distribution at short time (1-2 days and one week after transplantation donor cells were labeled with fluorescent dye PKH26 (Sigma) following manufacturer instruction.

As controls, **nmd** and wild-type mice were injected with vehicle by the same surgical procedure.

**Neuromuscular evaluation and survival**

Transplanted mice were observed daily for survival. A corresponding number of transgenic littermates was used as controls. Motor function was tested by using an accelerating rotarod device (4–40 r.p.m. Rota-Rod 7650; Ugo Basile, Comerio, Italy). The time during which mice remained on the rotarod was registered.

Mortality was scored as the age at death, when the mouse was unable to right itself within 30 s when placed on its back in a supine position (59).
Motor neuron and axon count

Paraffin serial sections (12.5 µm) of lumbar spinal cord were processed for Nissl-staining quantification of motor neuron numbers on the light microscope as described (60). For axon count, the tissue was dissected, immersed in 2.5% glutaraldehyde overnight and then post-fixed in 2% osmium tetroxide. Samples were then dehydrated in ethanol and embedded in Epon. Semi-thin transverse sections (1 µm) were stained with toluidine blue. L4 roots were examined for axon counting on the optic microscope.

Tissue analysis

The animals were sacrificed, perfused and fixed with 4% PFA in PBS (pH 7.4). The spinal cord was isolated, immersed in PFA solution for 1 hour, then in sucrose 20% solution in PBS (pH 7.4) overnight and frozen in Tissue Tek OCT compound with liquid nitrogen. The tissues were cryosectioned and mounted on gelatinized glass slides. Every 10th section 20 µm was collected. All sections were blocked with 1% foetal calf serum in PBS and permeabilized with 0.25% Triton X-100.

Sections were processed for multiple markers to determine the cellular phenotype of GFP/YFP-labeled cells. Primary antibodies were added overnight at 4°C at dilutions of 1:200 for Neu-N (mouse monoclonal antibody, Chemicon), 1:200 for NF (mouse monoclonal antibody, Chemicon), 1:200 for TuJ1 (mouse monoclonal antibody, Chemicon), 1:200 for MAP2 (mouse monoclonal antibody, Sigma-Aldrich), 1:200 for nestin (mouse monoclonal, Chemicon), 1:200 for vimentin (mouse monoclonal, Chemicon), 1:100 for rabbit anti-ChAT (Chemicon); rabbit anti-Islet-1 (1:200; Chemicon), rabbit anti-HB9 (1:200; Chemicon), 1:500 for rabbit anti-tyrosine hydroxylase (TH, Chemicon), 1:100 for anti GAD 67 (mouse monoclonal, Chemicon), 1: 100 goat α-Doublecortin
(DCX C-18, Santa Cruz Laboratories), 1: 200 oligodendrocyte marker O4 (mouse monoclonal, Chemicon), 1:200 for glial fibrillary acidic protein (mouse monoclonal Cy3 conjugate, Sigma).

For secondary antibodies: donkey -goat, mouse, rabbit or rat conjugated with fluorescein (FITC), R-PE, CY3 or biotin, 1:200 (Jackson Immuno Research and DAKO) were used for 1 hour at RT as secondary antibody, when unconjugated primary antibody was used.

Anti-GFP antibody rabbit serum Alexa 488 (1:400 dilution; Molecular Probes) was used to reveal GFP/YFP positivity in double immunostaining.

Co-expression of GFP/YFP and tissue specific markers was evaluated by conventional fluorescence microscope (Zeiss Axiophot, Germany) and by laser confocal scanning (Leica TCS SP2 AOBS, Germany) microscopic analysis.

To obtain an unbiased sterological estimate of YFP positive cells, optical disectors and random sampling were used. For donor cells quantification, a systematic random series of every 10th coronal section (20 µm) were obtained throughout the entire spinal cord (a mean of 25 sections for animal).

Numerical density of neurons was then estimated using the optical dissector method (61, 62). Optical disectors sized 100 X 70 X 14 µm were randomly sampled and the number of positive cells in each dissector was quantified. The density was calculated dividing the total number of YFP cells by the total volume of optical disectors. The total volume of tissue per specimen \( V_{\text{cord}} \) containing labelled neurons was calculated using the Cavalieri method. This total volume of tissue, multiplied by the number of neurons per µm³, gave the total number of neurons per specimen \( N=N_v\times V_{\text{cord}} \) (62).

Immunohistochemistry for GFAP, O4 and doublecortin with FISH was performed as described (63).

**RNA Extraction and Microarray**
Microarray data is in compliance with the Minimal Information About a Microarray Experiment (MIAME) format. All primary microarray data are available at GEO web site http://www.ncbi.nlm.nih.gov/geo/; GEO accession number: GSE3075.

We analyzed the lumbar spinal cord tract of transplanted nmd mice (n=3), untransplanted nmd mice (n=3) and wild-type mice (n=3) (all mice are of male gender). The latter groups underwent surgical procedure with vehicle. The surgical procedure and cell transplantation were done at P1 and the animals were sacrificed at 6 weeks of age.

Frozen tissue was stored at -80°C. Total RNA was purified from tissue, using the EUROZol Kit (EuroClone Ltd., United Kingdom) following the manufacturer’s instructions. Total RNA (5 μg) was reverse-transcribed with a cDNA synthesis kit in the presence of SuperScript II RT (Invitrogen-Life Technologies, Inc.) and an oligo dT-T7 primer (Affymetrix Inc., Santa Clara, CA). After phenol/chloroform extraction and ethanol precipitation, the cDNA pellet was air-dried and re-suspended in 12 µL of diethyl pyrocarbonate-treated water and quantified by spectrophotometer measurement. An aliquot of each sample was conserved for gel electrophoresis and for inspection of RNA quality (i.e., 28S:18S RNA ratio). 10 µl was used for the IVT (In Vitro Transcription) amplification reaction, in the presence of biotinylated nucleotides (Enzo Biochem Inc.). Labeled cRNA (15 µg) was fragmented by incubation at 94°C for 35 minutes in fragmentation buffer (GeneChip® Sample Cleanup, Qiagen) and the fragmented cRNA was then hybridized competitively against the Affymetrix 430 2.0 Array oligonucleotide array. Hybridization occurred at 47°C overnight. The arrays were scanned using a GeneArray® 2500 Scanner (Affymetrix) and analyzed using MicroArray Suite 5.0 (Affymetrix). All experiments were validated after direct measures of cRNA quality: 5’:3’ ratios for β-actin and for GAPDH (determined from Affymetrix chip hybridization analysis) are indirect indicators of overall RNA preservation. The default parameters were used for the statistical algorithm and for probe set scaling. The target intensity was set to 1,000 to normalize expression levels across all samples.
Data Analysis

The microarray data derive from three different groups: wild-type controls, mutant *nmd* and transplanted *nmd* mice. Each population consists of three RNA profiling samples. Our analysis is based on three outputs of Affymetrix GeneChip arrays: "Signal" (expression level), "Difference" and "Detection" (absent/present) calls were generated for each gene. Subsequent analyses used Microsoft Excel (Microsoft Office® package) and in-house analytical and statistical tools.

Only probes with the majority of transcripts (2 out of 3) in any group were considered. Almost 51% of the sequences passed this first screening. Means of signal expression for treated (MT), mutated (MM) and control (MC) groups were used to compute three log ratios; log2(MM/MC), log2(MT/MM) and log2(MT/MC). A second selection criterion required that at least one of these three log ratios had an absolute value greater than 1.5. 138 elements were retained (0.6% of total number in Affymetrix GeneChip).

Every probe was associated with its NCBI identifier, tissue of expression and gene name in order to correlate every cluster with characteristics determining a biological homogeneity. Matlab software was used to develop and perform the calculation routines. Functional grouping of genes was based on annotation from LocusLink, OMIM, sequence homology analysis, Information Hyperlinked over Proteins (iHOP) (64) and literature searches.

Quantitative Real-Time RT PCR analysis

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) data were obtained as follows: 1.5 μg of total RNA from each single mouse was reverse-transcribed by Ready-To-Go according to the manufacturer's recommendations (Amersham Pharmacia Biotech). The generated first-strand cDNA was diluted and used as template for Real-Time quantitative PCR analysis using Assays-on-Demand™ Gene Expression products (Applied Biosystems, Foster City, CA). This method uses a mix of unlabeled PCR primers and TaqMan® MGB probes (FAM™ dye-labeled) in
which the target sequence is located within the primer-generated amplicon. The reporter probe has a fluorescent dye on the 5'-end (FAM) and a quencher on the 3'-end (TAMRA). During the extension phase of the PCR amplification, the 5' exonuclease activity of the Taq DNA polymerase cleaves the probe releasing the fluorescent dye from the quencher. At each cycle of the PCR process, the increase in fluorescence was monitored by an ABI PRISM® 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems). The following gene expression assays were detected: Dnmt3a (Mm00432870-m1), Igfbp5 (Mm00516037), Slc1a3 (Mm0060697-m1, Cap1, (Mm00482950), Pvalb (Mm00443100), Ighmbp2 (Mm00456315_m1), Fxyd6 (Mm00445583-m1).

The expression level of the 18S ribosomal RNA gene (Hs99999901_s1) was used to normalize for differences in input cDNA. Each gene was profiled separately, but the same pool of cDNA was used for all reactions. For each gene (comprising the 18S), we used commercially available pre-developed TaqMan assays (PE/AB). PCR primers for each gene were designed in adjacent exons and the fluorogenic probe spanning the junction between them. PCR reactions occurred in a volume of 20µl, in which 1µl of diluted cDNA solution was used as template for each reaction. Thermal cycling program was first set at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 s and 60°C for 1 min. All experiments were performed in quadruplicate, and several negative controls were included. The data measured by quantitative Real-Time RT-PCR analysis were analyzed by Student’s t tests.

**Western blot**

20 µg of protein extract from lumbar spinal cord tract of transplanted *nmd* mice, untransplanted *nmd* mice and wild-type mice at 6 weeks of age, were separated on a 9% acrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The blots were probed for expression of doublecortin. Secondary peroxidase-conjugated antibodies were used and the signal was detected with an ECL detection kit (Amersham, Buckinghamshire, England). Murine brain of E.13.5 was used as positive control.
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Legends to Figures

Figure 1

Identification, isolation and differentiation of ALDH$^{\text{hi}}$SSC$^{\text{lo}}$ cells from spinal cord

A-B: Representative flow cytometric analysis of ALDH activity on spinal cord derived cells. NSCs were selected according to forward scatter (FSC) and side scatter (SSC) properties. NSC cells incubated with Aldefluor substrate and the specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), were used to establish the baseline fluorescence of these cells and to define the ALDH$^{\text{hi}}$ region (A). Incubation of NSC cells with Aldefluor substrate in the absence of an inhibitor induced a shift in FL1 fluorescence defining the ALDH$^{\text{hi}}$ population (B).

C-E Sorted ALDH$^{\text{hi}}$SSC$^{\text{lo}}$ cells grown at clonal density in neuroepithelial (NEP) medium form nestin-positive epithelial-like clones. Panel C illustrates one of these colonies stained for nestin (red signal). Furthermore these cells express gene characteristic of neural and other stem cells, including Sox2 (D, red signal) and musashi-1 (E, green signal). After in vitro differentiation, ALDH$^{\text{hi}}$SSC$^{\text{lo}}$ cells give rise to neurons with complex morphology. ALDH$^{\text{hi}}$SSC$^{\text{lo}}$ cells from YFP transgenic mice that express YFP protein (green) only in neurons produce neuronal cells expressing neuronal markers such as TuJ1 (G: TuJ1; H: YFP; I: merge). ALDH$^{\text{hi}}$SSC$^{\text{lo}}$ cells, after in vitro priming, generate neurons positive for motor neuronal marker Islet-1 (F: green nuclear signal, wild type cells) and HB9 (J: HB9; K: YFP; L: merge).

Nuclei are counterstained with DAPI (blue signal).

Scale bar: C: 100 µm; D-E: 50 µm; F: 60 µm; G-L: 80 µm.

Figure 2

ALDH$^{\text{hi}}$SSC$^{\text{lo}}$ derived motor neurons present long-neuritic extension and form neuromuscular junctions.
After *in vitro* priming and differentiation, Thy1-YFP ALDH\(^{hi}\)SSC\(^{lo}\) cells acquire a differentiated phenotype extending long axons in the culture plate (A, B). These cells express neuronal antigens as shown in C, where MAP2 signal (red) colocalizes with YFP green signal (merge yellow signal). A fraction of YFP neurons (E-H) present a cholinergic phenotype, as confirmed by the expression of ChAT (D and G, merge: F and I) and generate neuromuscular junctions (J-L) in co-culture with C2C12 myotubes.

Confocal tridimensional microscopy demonstrate the co-localization of YFP\(^+\) axons (K) and α-bungarotoxin-stained acetylcholine receptors (J, merge: L). Nuclei are stained with DAPI.

Scale bar: A-B: 100 µm; C-I: 80 µm; J-L: 100 µm.

**Figure 3**

**ALDH\(^{hi}\) SSC\(^{lo}\) cells transplanted in nmd mice survive and generate motor neurons.**

After intratechal transplantation of Thy1-YFP derived ALDH\(^{hi}\)SSC\(^{lo}\) cells into *nmd* mice, we detected YFP\(^+\) neurons in the gray matter of the spinal cord over the rostral caudal length and cervical and lumbar enlargement. In particular YFP\(^+\) cells (green signal) were detected in the anterior horn of the spinal cord as shown in spinal cord coronal sections (A-B). Furthermore YFP\(^+\) axons (green signal) are detected within ventral roots, labeled with NF (red signal), of transplanted *nmd* mice as shown in C (merge yellow signal).

Immunohistochemistry for neuroectodermal markers confirm that these cells are differentiated in neurons. Confocal microscopy showed that YFP donor-derived neurons (E) co-express neuronal specific proteins such as TuJ1 in D, F: merge, the corresponding movie is provided as supplementary data, movie 1) and NeuN in G.

To test alternative non-neuronal fates of transplanted cells, immunohistochemistry for glial antigens (GFAP) combined with FISH analysis for Y chromosome in sex-mismatched transplantation (male donor cells in female recipient) was performed, demonstrating the presence of donor-derived GFAP\(^+\)Y\(^+\), as shown in panel H.
I-L: A fraction of YFP neurons present motoneuronal features as demonstrated by double immunofluorescence staining and three-dimensional confocal reconstruction of YFP (green) and the motoneuronal marker HB9 (red) (I: merge), and cholinergic neurotransmitters as in panels J-L (J: ChAT; K: YFP; L: merge).

Scale bar: A: 300 µm; B: 100 µm; C: 70 µm; D-G: 50 µm; H: 50 µm; I-L: 60 µm.

**Figure 4**

**Phenotype characterization of transplanted nmd mice**

A: Neonatal-to-weaning growth curve and mean body weight of treated and untreated mice compared with unaffected littermate controls (Data represent mean ± SD). Transplanted nmd mice displayed an improved growth rate untreated nmd mice.

B: Rotarod test of treated and untreated nmd mice and wild-type mice (Data represent mean ± SD).

C: Survival (Kaplan–Meier) analysis of nmd-transplanted mice. Survival was significantly extended for transplanted mice compared untreated mice.

D: Suspended by the tail, untreated nmd mice (on the left, movie 2) presented dorsally contracted hindlimbs and were not able to pull themselves up from the ground, compared to treated nmd mice (in the middle, movie 3) and wild-type littermates (on the right, movie 4). The corresponding movies are provided as supplementary data.

**Figure 5**

**Spinal cord and ventral root analysis**

A-C: Histology of lumbar cord in nmd treated (B) and untreated (C) mice and wild type mice (A).

D-F: Nissl-staining of motor neurons in the lumbar spinal cord from 6-week-old treated (E) and untreated (F) and wild-type (D) mice.

G-I: Light microscope images of L4 ventral roots from wild-type (G) mice, transplanted (H) mice and mutant (I) mice at 6 weeks of age. Note the reduction of axonal density in the L4 ventral nerve
root from a \textit{nmd} mouse (B) compared to a control ventral root. Significant improvement is observed in L4 of treated \textit{nmd} mice.

J-K: Motor neuronal count in the lumbar spinal cord of treated and untreated \textit{nmd} mice and wild-type (Data represent mean ± SD), at 6 weeks of age (J) and at the final end stage of the disease (K).

L-M: Quantification of axons in wild-type, mutant and transplanted mice (Data represent mean ± SD) at 6 weeks of age (L) and at the final end stage of the disease (M).

Scale bar: A-C: 250 μm; D: 60 μm; E-F: 30 μm; G-I: 100 μm.

\textbf{Figure 6}

\textbf{Doublecortin expression in spinal cord of \textit{nmd} mice}

A) Confocal analysis of transplanted \textit{nmd} spinal cord showing the presence of cells double positive for doublecortin (red signal) and YFP (green signal).

B) Immunohistochemical analysis for DCX associated with FISH for Y chromosome in sex mismatched transplantation (female donor and male recipient) demonstrating the presence of DCX⁺Y⁺ newly generated neurons in \textit{nmd} treated spinal cord.

(DCX: purple; Y chromosome: red dot; Nuclei counterstained with DAPI: blue).

C) Western blot analysis of doublecortin in spinal cord of wild-type (lanes 1, 2), untreated \textit{nmd} (lanes 3, 4) and treated \textit{nmd} (lanes 5, 6, 7) mice, demonstrating the presence of doublecortin in spinal cord of transplanted animals. Murine brain of E.13.5 was used as positive control (lane 8).

Scale bar A-C: 80 μm; D-F: 60 μm.
Table 1. Summary chart of enrichment profile for neural stem cells after FACS analysis

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<tr>
<th>Source of cells</th>
<th>ALDH$^{hi}$SSC$^{lo}$ cells (as % of all cells)</th>
<th>Stem cell frequency in ALDH$^{hi}$SSC$^{lo}$ cells</th>
<th>ALDH$^{hi}$SSC$^{lo}$ cells positive cells in secondary clones</th>
<th>Stem cell frequency in negative fractions</th>
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Table 2 Top up-regulated (A) and down-regulated (B) genes in the lumbar spinal cord of mutated *nmd* mice vs. wild-type mice

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Table 3 Top up-regulated (A) and down-regulated (B) genes in the lumbar spinal cord of treated nmd mice vs. wild-type and untreated nmd mice

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<tr>
<th>Gene name</th>
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Fig. 2
Fig. 4

A

B

C

D

Downloaded from http://hmg.oxfordjournals.org/ by guest on April 26, 2016
Fig. 5

The figure consists of several panels labeled A to M, each containing microscopic images or bar charts. The images appear to be histological sections, possibly of neural tissue, with varying structures and staining. The graphs represent quantitative data, likely related to neural counts or measurements, with categories such as 'unr-nmd', 'tr-nmd', and 'control'.
Fig. 6