Altered in vitro Proliferation of Mouse SOD1-G93A Skeletal Muscle Satellite Cells

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Key Words

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Neurodegenerative disease • SOD1 mutants • Satellite cell •
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Abstract

Background: Amyotrophic lateral sclerosis (ALS) is the most common adult-onset neurodegenerative disease characterized by ascending muscle weakness, atrophy and paralysis. Early muscle abnormalities that precede motor neuron loss in ALS may destabilize neuromuscular junctions, and we have previously demonstrated alterations in myogenic regulatory factor (MRF) expression in vivo and in the activation of myofiber-associated skeletal muscle satellite cells (SMSCs) in the mouse model of ALS (SOD1-G93A). **Methods:** To elucidate niche dependence versus cell-autonomous mutant SOD1 (mSOD1) toxicity in this model, we measured in vitro proliferation potential and MRF and cyclin gene expression in SMSC cultures derived from fast-twitch extensor digitorum longus and slow-twitch soleus muscles of SOD1-G93A mice. **Results:** SMSCs from early presymptomatic (p40) to terminal, semi-paralytic (p120) SOD1-G93A mice demonstrated generally lower proliferation potential compared with age-matched controls. However, induced proliferation

was observed in surgically denervated wild-type animals and SOD1-G93A animals at p90, when critical denervation arises. SMSCs from fast and slow muscles were similarly affected by mSOD1 expression. Lowered proliferation rate was generally corroborated with decreased relative MRF expression levels, although this was most prominent in early age and was modulated by muscle type origin. Cyclins controlling cell proliferation did not show modifications in their mRNA levels; however, the expression of cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), which is known to promote myoblast differentiation, was decreased in SOD1-G93A cultures. **Conclusions:** Our data suggest that the function of SMSCs is impaired in SOD1-G93A satellite cells from the earliest stages of the disease when no critical motor neuron loss has been described.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal neurodegenerative disease. The main hallmark of ALS is a selective death of motor neurons in the brain and spinal cord ultimately leading to the paralysis of voluntary muscles. Dysfunction of upper motor neurons causes

spasticity and hyperreflexia, whereas impairment of lower motor neurons triggers generalized muscle weakness, atrophy and paralysis [1]. Whereas the great majority of the ALS cases are considered as sporadic, about 10% of the cases are familial (fALS) with identified heritable genetic component. Approximately 10–15% of fALS are caused by mutations in *Cu/Zn superoxide dismutase (SOD1)* gene [2]. A mouse model of ALS that overexpress glycine 93 to alanine (G93A)-mutated human Cu/Zn superoxide dismutase (mSOD1 or SOD1-G93A) and develops motor neuron pathology similar to that observed in human fALS [3–5]. As mSOD1 can induce motor neuron apoptosis, it was hypothesized that its expression uniquely in these cells was sufficient to cause ALS. However, this theory has been questioned as targeted expression of mSOD1 specifically in motor neurons does not always lead to an ALS phenotype [6, 7]. Indeed, in chimeric animals harboring different proportions of cells expressing wild-type and mSOD1, the number of wild-type SOD1 expressing cells was positively associated with lifespan whether these cells were motor neurons or not [for review see 8]. In agreement with these findings, other tissues have been shown to be implicated in ALS; astrocytic activation and secretion of proteins toxic for motor neurons has been described in ALS patients [9] and ablation of microglial and macrophage lineage increases lifespan of mSOD1 mice [10]. Muscle cells may also play an active role in the pathobiology of ALS as one of the earliest signs of the disease is altered muscle metabolism [11, 12], followed by disruption of the neuromuscular junction and subsequent retrograde axonal degeneration leading to the motor neuron death [13]. Muscle alterations, such as increased nutrient uptake, modified carbohydrate, lipid metabolism and altered mitochondrial uncoupling and respiration take place before the first signs of motor neuron degeneration [12, 14, 15]. Therefore, certain muscle abnormalities seem to precede motor neuron death rather than resulting from it. Expression of insulin-like growth factor-1 (Igfl) specifically in skeletal muscle delays the disease in mSOD1 mice [16], and it was proposed that the effect of Igfl is mediated through the activation of skeletal muscle satellite cells (SMSCs), committed muscle progenitors present in the periphery of adult muscle fibers [17]. In adults, SMSCs remain quiescent, expressing the satellite cell marker paired-box transcription factor *Pax7* [18–21]. Upon distinct stimuli such as acute injury, exercise or muscle denervation, they activate [22], re-enter the cell cycle and start proliferating to coexpress *Myod1* (myogenic differentiation 1) [23–25]. Subsequently, SMSCs up-regulate *myogenin* and elongate to form

new myotubes to regenerate muscle [22, 26]. *Myod1* and *myogenin* are known as myogenic regulatory factors (MRFs). These MRFs are muscle-specific members of the basic helix-loop-helix (bHLH) transcription factor superfamily [27] that heterodimerize with bHLH transcription factors named E proteins to drive the expression of key muscle genes such as muscle creatine kinase, troponin I, acetylcholine receptor α -subunit and myosin heavy and light chains [28, 29]. Recent work has described an abnormal senescent-like morphology and reduced myosin heavy chain isoform expression in myotubes obtained from in vitro myoblast cultures established from ALS patient-derived muscle biopsies [30]. The authors concluded that in ALS patients, SMSCs' ability to differentiate is severely impaired. Consistently, evidence recently published by us also suggests that MRF expression is altered in the skeletal muscle of SOD1-G93A mouse model [31], and that satellite cell number and activation status are altered [32]. Importantly, fiber type- and age-related differences were also reported [32].

Here, we investigated the proliferative potential and *Pax7*, MRF and cyclin gene expression in satellite cell cultures from extensor digitorum longus (EDL), mainly composed of fast-twitch myofibers (type II) and soleus (SOL), with slow-twitch myofibers (type I), from the early presymptomatic to the terminal phase of the disease in SOD1-G93A mice. We observed a consistent diminution in the SOD1-G93A SMSCs' ability to proliferate in both muscles under study and at all stages except in 90-day-old SMSC cultures where an increment existed. *Pax7* and MRF expression was also modified according to proliferative potential except in the latest phases of the disease when a deregulation of these myogenic program factors was observed.

Materials and Methods

All experimental procedures were approved by the Ethics Committee of the University of Zaragoza, and followed the international guidelines for the use of laboratory animals. Mice were housed under a 12-hour light/dark cycle at 21–23°C with relative humidity of 55%. Food and water were available ad libitum. Animals were sacrificed by cervical dislocation.

Satellite Cell Extraction and Culture

Three male B6SJL and SOD1-G93A mice per age group were sacrificed at the early presymptomatic (p40), late presymptomatic (p60), symptomatic (p90) and terminal stages (p120). EDL (fast-twitch) and SOL (slow-twitch) muscles were collected and processed in parallel. Muscle satellite cell extraction and culture were carried out following the described protocol [33, 34]. Pooled muscles per age and muscle type were minced to a slurry and

digested in a solution of 0.1% trypsin and 0.1% collagenase (w/v; Sigma-Aldrich) for 30 min at 37°C. The enzymatic reactions were stopped with 20% fetal bovine serum (Gibco) diluted in DMEM + GlutaMAX (Gibco), and three to four rounds of digestion were performed until the muscle bulk was digested completely. The cell suspension from each group was filtered through a 70- μ m diameter sterile strainer and centrifuged at 1,800 rpm for 15 min at 4°C. Cell pellet was resuspended in DMEM + GlutaMAX (Gibco) and stained with 0.4% trypan blue (Sigma-Aldrich) for viable cell counting. Cell proliferation and gene expression analysis were carried out in freshly isolated (passage zero) cells to avoid the possibility that mSOD1 SMSCs display altered susceptibility to freezing or trypsinization. For proliferation and gene expression analysis, a total of 1,000 cells were seeded in each well of a 96-well plate coated with Matrigel basement membrane matrix (Becton Dickinson SA). The culture medium was composed of 39% F-12 + GlutaMAX (Gibco), 39% DMEM + GlutaMAX (Gibco), 10% fetal calf serum (Gibco) and 2% Ultrosor G (Pall-Biosepra). Cells were left to adhere and start proliferating for 3.5 days at 37°C and 5% CO₂. At least four replicate wells were plated for each group of muscle type and age. Experiments were performed in parallel for cell proliferation and gene expression analysis.

Cell Proliferation Assay

Starting 3.5 days after the plating, and repeatedly every 24 h until 7.5 days, cell proliferation plate was fixed with 10% neutral buffered formalin solution (Sigma-Aldrich) for 15 min, and nuclei were stained with Hoechst 33342 (Bis Benzimide H 33342 trihydrochloride, Sigma-Aldrich) for 5 min. After fixation, cells were washed with 1 $\times$ PBS, and five random fields per well were photographed under an epifluorescence microscope (Nikon TE2000-E) at $\times$ 20 magnification and 325 nm. For each time point, the total number of nuclei per replicate (total 20 fields) was manually counted for each group of muscle type and age. For each day of culture, data were compared with the corresponding muscle and age group of interest (see below for the statistics). Data of the four replicates were averaged and normalized setting the maximum value from each comparison to 100. Error bars represent the standard error between replicates as percentage of the maximum value of the comparison that has been set to 100.

Quantification of Gene Expression

Pax7 and *Myod1* genes were analyzed as markers for satellite cell determination and activation, respectively, and *Pcna* and *Ccnd1* genes were analyzed as markers of cell cycle entry, at 5.5 days of culture when proliferation was prominent without significant differentiation [21, 35]. The expression of cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), a known target for *Myod1* and *myogenin* and promoter of satellite cell differentiation, was also quantified [36]. Finally, *myogenin* mRNA, a marker of differentiation process, was measured at day 7.5 when clear myotube formation was observed.

Cells were washed with cold 1 $\times$ PBS and transferred to -80°C to wait for RNA extraction and cDNA synthesis. The plates were processed according to the instructions of Cells-to-cDNA kit (Ambion) instructions. Plates were thawed on ice, and cells were lysed by adding 100 μ l of cell lysis solution. To release RNA from cells and eliminate endogenous RNase activity, lysed cells were

subjected to heat treatment for 15 min at 75°C. Traces of genomic DNA were eliminated with Dnase treatment at 37°C for 15 min, followed by 5 min of heat inactivation at 75°C. cDNA synthesis reactions from each RNA sample were performed in duplicates as follows. Each reaction included a pool of 2 μ l of dNTPs, 1 μ l of random hexamers, and 5 μ l of RNA and was incubated at 75°C for 3 min. Subsequently, 1 μ l of 10 $\times$ reverse transcription buffer, 0.5 μ l M-MLV retrotranscriptase and 0.5 μ l RNase inhibitor were added and the reaction was incubated at 42°C for 60 min and then at 95°C for 10 min. For QPCR, cDNA was diluted 1:10 in dH₂O, and 2 μ l were used as a template for each reaction (3 replicates per cDNA sample) containing 2.5 μ l Fast 2 $\times$ TaqMan master mix (No AmpErase UNG) and 0.5 μ l gene-specific TaqMan assays (Applied Biosystems) for *Pax7* (Mm00834079_m1), *Myod1* (Mm00440387_m1), *Pcna* (proliferating cell nuclear antigen)(Mm00448100_g1), *Ccnd1* (cyclin d1)(Mm00432359_m1), *Cdkn1a* (cyclin-dependent kinase inhibitor 1A)(Mm00432448_m1) or *myogenin* (Mm00446194_m1) in a final volume of 5 μ l. Reactions were run using StepOne Plus Real-Time PCR System (Applied Biosystems) using the following conditions; 95°C for 10 min and 47 cycles of 95°C for 15 s and 60°C for 30 s. In each cDNA sample, three endogenous reference genes were amplified using TaqMan assays: 18S ribosomal RNA (4352930E), *Gapdh* (4352932E), and β -actin (4352933E). Target gene expression results were normalized using geometric mean of these three housekeeping genes [37–39], and relative gene expression was determined using the 2- $\Delta\Delta$ CT method and the data from the wild-type derived cultures for each group as calibrator [40]. For all primer/probe sets, reaction efficiencies approached 100%.

Muscle Denervation Procedure

Six male mice (B6CLJ strain) at p60 were anaesthetized (pentobarbital 30 mg/kg, i.p.), and the right leg muscle denervation was performed by extraction of a 5-mm segment of the sciatic nerve through an incision in the mid-posterolateral area of the thigh. The left limb remained unoperated, and was used as a control. The incision was closed with silk sutures and washed daily with antibacterial solution to prevent infection. The absence of toe-spread reflex was confirmed daily. Fifteen days after surgical denervation (age p75), the animals were sacrificed by cervical dislocation, and control and denervated EDL and soleus muscles were harvested and processed as described above.

Immunofluorescence

One well per group was fixed and immunofluorescence for MYOD was performed in order to ensure the myogenic origin of the cells. Cells were rinsed with PBS and fixed in neutral buffered formalin solution 10% (Sigma-Aldrich) for 10 min at room temperature. Permeabilization was performed by incubating cells in a solution composed of 0.2% (v/v) Triton X-100 (Sigma-Aldrich) and 50 mM NH₄Cl for 10 min and saturation for 10 min with 0.2% (w/v) gelatine in PBS. Primary antibody for MYOD (sc-304, Santa Cruz Biotechnology) was added 1:100 in PBS for 2 h at room temperature and visualized with Alexa 546-conjugated anti-rabbit secondary antibody 1:300 (Invitrogen). Nuclei were stained with 1,000 ng/ml Hoechst 33342 (Sigma-Aldrich) and mounted in Fluorekeep mounting medium for immunofluorescence (Argene). All cultures used in this study showed a MYOD-positive content of 70–80%.

Fig. 1. Proliferation and differentiation process of mouse myogenic cell cultures. **a** Photographs show the in vitro appearance of satellite cell cultures 3.5, 7.5 and 10 days after plating. **b** The myogenicity of the cells was assessed 4.5 days after plating by costaining for Hoechst and immunofluorescence analysis for MYOD. MYOD-positive cells at this stage represented 70–80% of the population.

Statistical Analysis

In the proliferation assay data analysis, total cells from five photographic fields of a single replicate well were manually counted, and the mean and standard error of mean for each group was calculated from the value of four replicate wells (total 20 fields per data points). For the relative quantitative PCR, statistical analysis was performed on the data obtained from the two cDNA synthesis reactions from each well and two biological replicate wells (total 4 data per group). Results obtained from SOD1-G93A and control groups were compared using Student's *t* test (Statistica 5.0, Statsoft software) [31]. Statistical differences were considered significant at $p < 0.05$.

Results

To assess the myogenic origin of the cells, cultures were maintained for 10 days in conditions that supported both proliferation and differentiation; fully developed myotubes were observed at the end of this period (fig. 1a). Additionally, based on immunostaining for MYOD at 4.5 days, all cultures used in the experiments showed 70–80% MYOD-positive cells (fig. 1b).

Despite a slight tendency at advanced culture days, no clear proliferative differences were observed between wild-type and SOD1-G93A in the case of EDL-derived SMSCs cultures at p40 (fig. 2a). By contrast, SOL-derived SMSCs from SOD1-G93A mice at p40 proliferated slower than their wild-type littermates (fig. 2b). At late presymptomatic day p60, SOD1-G93A cells proliferated slower than wild-type SMSCs in both muscles under study (fig. 2c, d). Surprisingly, at symptomatic day p90, SMSCs derived from both EDL and SOL muscles proliferated faster than those obtained from control littermates, although the substantial sample variation precluded significance in the case of EDL (fig. 2e, f). However, SMSCs from animals of 120 days of age, where muscle atrophy is already severe, proliferated again slower than their age-matched wild-type SMSCs (fig. 2g, h).

To depict to what extent denervation influenced SMSCs proliferation, surgically denervated wild-type mice SMSCs were analyzed. SMSCs derived from EDL and SOL muscles of denervated limb displayed a superior proliferation rate compared to the SMSCs derived from

Fig. 2. Growth curves from wild-type and SOD1-G93A mouse satellite cell cultures. Pairwise comparison of the satellite cell culture proliferation curves obtained from SOD1-G93A and wild-type muscles at p40 (early presymptomatic stage) EDL (**a**) and SOL (**b**), p60 (late presymptomatic stage) EDL (**c**) and SOL (**d**), p90 (symptomatic stage) EDL (**e**) and SOL (**f**) and p120 (terminal stage) EDL (**g**) and SOL (**h**), and from denervated EDL (**i**) and SOL (**j**) muscles. * $p < 0.05$, ** $p < 0.01$, Student's *t* test.

Fig. 3. Pax7, Myod1, Cdkn1a and myogenin expression in satellite cell cultures. Pax7 (a and b), Myod1 (c and d), Cdkn1a (e and f) and myogenin (g and h) levels in proliferating and differentiating satellite cells from EDL (a, c, e, g) and SOL (b, d, f, h) muscles. In each panel, relative expression in cells from SOD1-G93A (light grey bars) and age-matched wild-type mice (black bars) at p40, p60, p90 and p120 are shown. Additionally, expression in denervated compared with intact muscles from the same animal are shown. Analysis was performed 5.5 (a, b, c, d, e and f) and 7.5 days (g and h) after plating. * p < 0.05, ** p < 0.01, Student's t test.

the unoperated limb (fig. 2i, j). This supports the view that gross denervation at the onset of the disease symptomatic stage (p90) in SOD1-G93A mice may have stimulatory effects on SMSC proliferation rate.

Although all data points did not reach significance, the overall conclusion is that SMSCs derived from SOD1-G93A mice proliferate at a lower rate compared to their wild-type-controls. This tendency is maintained from the early presymptomatic to the terminal stages of the disease in both muscle types studied. However, at the symptomatic stage, the proliferation rate is increased in mSOD1 SMSCs, a result that parallels the one observed in SMSCs derived from denervated muscle. It is of interest to note that even if the fast and slow muscles are unequally affected in the mice, the in vitro proliferation of SOD1-G93A SMSCs derived from both types of muscles are similarly affected compared to the age-matched wild type.

To get insight into the molecular mechanisms that may drive the observed impaired proliferative potential in SOD1-G93A satellite cells, we analyzed the expression of MRFs and cyclins implicated in the satellite cell determination and myogenic program (see Materials and Methods). At p40, SOD1-G93A SMSCs derived from EDL muscles presented a diminution in the levels of *Pax7* mRNA (fig. 3a, $p < 0.05$) compared to wild-type cells. This result was correlated with decreased *Myod1* (fig. 3c, $p < 0.05$), *Cdkn1a* (fig. 3e, $p < 0.01$) and *myogenin* mRNA levels (fig. 3g, $p < 0.01$). In SOL-derived SMSCs, similar tendency to diminution in the levels of *Pax7* mRNA (fig. 3b), *Myod1* (fig. 3d) and *Cdkn1a* (fig. 3f) was accompanied with significantly reduced *myogenin* mRNA levels (fig. 3h, $p < 0.05$). Qualitatively similar results were obtained at the late presymptomatic stage (p60). Although *Pax7* diminution was only suggestive (fig. 3a), levels of *Myod1*, *Cdkn1a* and *myogenin* remained reduced in EDL SMSCs (fig. 3c, $p < 0.01$, fig. 3e, $p < 0.05$, and fig. 3g, $p < 0.01$, respectively). On the other hand, *Pax7* mRNA in SOL cultures was not altered (fig. 3b), and reduction in *Myod1*, *Cdkn1a* and *myogenin* was suggestive but non-significant (fig. 3d, f and h, respectively).

At the onset of symptoms (p90), when SOD1-G93A SMSCs displayed similar or relatively increased proliferation (fig. 2e, f), similar levels of *Myod1* mRNA were observed in mSOD1 SMSCs compared to the control in EDL (fig. 3c) and, in spite of the lower levels of *Cdkn1a* transcripts in SOD1-G93A cells (EDL, fig. 3e, $p < 0.05$ and SOL, fig. 3f, $p < 0.05$), *myogenin* expression reached wild-type levels in EDL (fig. 3g) and even showed tendency to increment in SOL (fig. 3h) cultures. This increase in acti-

vation and differentiation markers was accompanied with decreased SMSCs quiescence marker *Pax7* (EDL, fig. 3a, and SOL, fig. 3b, $p < 0.01$), being the downregulation of *Pax7*, an essential condition to induce differentiation of satellite cells [41, 42]. Finally, at the terminal stage (p120), a suggestive increment of *Pax7* and *Cdkn1a* and less pronounced in *Myod1* transcripts was observed in EDL-derived mSOD1 cells (*Pax7*, fig. 3a; *Myod1*, fig. 3c, and *Cdkn1a*, fig. 3e; $p < 0.01$); however, results showed a tendency to diminution of *myogenin* mRNA (fig. 3g). In mSOD1 SOL satellite cell cultures, *Pax7* transcripts remained as in controls (fig. 3b); however, *Myod1*, *Cdkn1a* and *myogenin* factors revealed a tendency or significant reduction in mutant cell cultures (*Myod1*, fig. 3d; *Cdkn1a*, fig. 3f, $p < 0.05$, and *myogenin*, fig. 3h). On the other hand, EDL-derived SMSC cultures from denervated limbs expressed higher levels of the factors analyzed, *Pax7*, *Myod1*, *Cdkn1a* and *myogenin* (*Pax7*, fig. 3a, $p < 0.01$; *Myod1*, fig. 3c, $p < 0.05$; *Cdkn1a*, fig. 3e, and *myogenin*, fig. 3g, $p < 0.01$). Nevertheless, SMSCs from denervated SOL cultures showed a tendency to decrease for *Pax7* (fig. 3b) and *Myod1* transcripts (fig. 3d, $p < 0.05$), but again *Cdkn1a* and *myogenin* were upregulated (fig. 3f, $p < 0.05$) or reached levels of wild-type cells (fig. 3h), respectively.

No significant differences were found in *Pcna* or *Ccnd1* expression between SOD1-G93A and wild-type cell cultures at any of the studied stages and muscles (fig. 4), except in the case of *Ccnd1* at p120 in EDL cultures, where SOD1-G93A cells, in coincidence with *Myod1* tendency, showed significant upregulation (fig. 4c, $p < 0.05$).

To summarize, SMSCs obtained from EDL and SOL muscles of SOD1-G93A mice cultured in vitro presented diminished levels of *Pax7*, MRFs and *Cdkn1a* mRNA at the early and late presymptomatic stages, although this was less obvious in SOL-derived SMSCs. The onset of symptomatic period coincided with increase to the wild-type level of *Myod1* and *myogenin*, although *Pax7* and *Cdkn1a* remained at relatively lower level. Finally, at the terminal stage, *Pax7* levels remained incremented especially in EDL-derived SMSCs. *Myod1* mRNA tended to increase in EDL and decrease in SOL, whereas *myogenin* transcripts tended to decrease in both SMSC cultures as in the earliest phases of the disease.

Discussion

In the SOD1-G93A model of ALS, the main signs of muscle pathology start at around 40 days of age with aggregation of mSOD1 proteins [43], loss of functional mo-

Fig. 4. *Pcna* and *Ccnd1* expression in satellite cell cultures. *Pcna* (a and b) and *Ccnd1* (c and d) levels in proliferating and differentiating satellite cells from EDL (a and c) and SOL (b and d) muscles. In each panel, relative expression in cells from SOD1-G93A (light grey bars) and age-matched wild-type mice (black bars) at p40, p60, p90 and p120 is shown. Additionally, expression in denervated compared with intact muscles from the same animal is shown. Analysis was performed 5.5 days after plating. * $p < 0.05$, ** $p < 0.01$, Student's t test.

tor units [44, 45] and reduction in the muscle contractile force [46]. The symptomatic stage starts at approximately 90 days of age with hind limb muscle weakness and fine tremors, and progresses to muscle atrophy and paralysis towards the terminal stage at approximately 120 days of age [47]. In pooled hind limb muscles, these changes are accompanied by transcriptional upregulation of MRF indicative of attempted regenerative response [31]. However, muscle fiber type differences have been described in the time course and severity of the mSOD1 muscle pathology [44, 45, 48]. We have recently described mSOD1-induced regenerative response of SMSCs in both fast and slow-twitch skeletal muscle fibers in situ [32]. However, whether the observed alterations in the number and activation of SMSCs pool in mSOD1 myofibers during the course of the disease arise from signals from the cellular niche or represent a cell-autonomous effect of mSOD1 to SMSCs function remains obscure. To this end, we inves-

tigated here the SMSCs proliferation and *Pax7*, MRF (*Myod1* and *myogenin*) and *cyclin* expression in SMSC cultures established from SOD1-G93A and wild-type mice from the phenotypically unaffected (p40) to the semi-paralytic terminal stage (p120). This enabled us to determine the capacity of these cells to proliferate and follow the MRF expression in the absence of trophic or inhibitory signals from the natural SMSCs niche as well as those derived from altered electrical stimulation by connected neurons. Because muscle type susceptibility to mSOD1 toxicity varies in vivo, in our study we used typical fast-twitch EDL composed of approximately half and half of fast glycolytic (type IIb) and fast oxidative-glycolytic (type IIa) fibers, as well as slow-twitch SOL containing approximately 60% slow oxidative (type I) fibers and 40% type IIa fibers [49].

In early presymptomatic as well as late presymptomatic stage cultures, a tendency towards lower proliferative

potential of SOD1-G93A satellite cells was observed in both muscles. On the other hand, diminished levels of MRFs and *Cdkn1a* were found, although sample variability prevented differences from being significant, especially in SOL-derived cultures. Our results parallel those of the earlier report of limited myogenicity in satellite cell cultures obtained from deltoid muscle biopsies in 7 ALS patients [30]. These authors observed an impaired myotube development in vitro, which is in agreement with our finding of decreased *myogenin* and *Cdkn1a* levels, both factors promoting satellite cell differentiation.

In our study, the onset of the symptomatic stage (p90) was associated with increased proliferation rate in mSOD1 carrying cells of both muscles under study. MRF gene expression roughly correlated with these findings: *Myod1* reached similar levels to wild-type cells, especially in EDL muscle, and *myogenin* shifted towards increment, especially in SOL-derived SOD1-G93A cultures. *Pax7* is an accepted satellite cell marker [18–21]; therefore, the increment of this cell population would be directly expected to lead to an increase in *Pax7* transcripts. However, this statement is not so straightforward, because *Pax7* downregulation is also necessary for satellite cell differentiation [41, 42]. Therefore, the observed *Pax7* transcript decrement in SOD1-G93A satellite cells during the symptomatic stage (p90) must be an interplay between the increased number of *Pax7*-expressing satellite cells and the downregulation of this factor to allow cell cycle exit and myoblast differentiation since by this stage, a critical loss of motor units and tetanic force and muscle disturbance has been described in both EDL and SOL muscles [13, 45, 50, 51]. Proliferation and turnover of SMSCs at high rate for at least one month after denervation has also been reported [52, 53]. Therefore, we suggest that denervation at the onset of the symptoms may temporarily stimulate satellite cell proliferation, and counteract the decreased proliferation rate caused by cell-autonomous mSOD1 toxicity observed at earlier phases of the disease. Finally, at the terminal stage (p120), a diminished proliferation capacity was observed for SOD1-G93A SMSCs of fast- and slow-twitch muscles. At the molecular level, *Pax7* showed tendency for increased mRNA expression, especially in EDL, possibly reflecting the grave denervation at this stage. However, whereas *Myod1* and *Cdkn1a* remained upregulated in EDL cultures, in SOL cultures they became downregulated, showing the heterogeneous myogenic response to denervation or mSOD1 insults. Finally, *myogenin* displayed marked tendency to be reduced in satellite cell cultures at this stage in agreement with the abortive myogenesis described in ALS pa-

tient-derived cells [30] and in denervated skeletal muscle [54].

Several factors may contribute to reduction in the proliferative potential of mSOD1 satellite cells. These include interactions between G93A-SOD1 and Bcl-2 through the cyclin regulator p27 and deregulation of cell cycle [55]. Additionally, mSOD1 aggregates may inhibit proteasomal machinery and deregulate cellular activities such as protein folding and organelle function (Golgi, endoplasmic reticulum, and mitochondria). On the other hand, mSOD1 impairment has been shown to induce oxidative stress and excessive and aberrant ROS chemistry [for review see 2], oxidative species being inhibitors of myogenesis [56–58]. Because *Ccnd1* and *Pcna*, both factors involved in early induction of DNA replication upstream MRF function [36, 59], were not significantly affected in mSOD1 cultures, it is likely that the disruption of the molecular events leading to mSOD1 satellite cell proliferation is located downstream those factors. Alternatively, considering that the regulation of *Pcna* and *Ccnd1* is mediated, in part, through the ubiquitin-proteasomal pathway [60], which has been shown to be altered in ALS patients and animal models [for review see 61], it is feasible that the modification of *Pcna* or *Ccnd1* function is a post-translational event in SOD1-G93A satellite cell cultures.

On the other hand, *Cdkn1a* is a promoter of cell cycle arrest; therefore, under physiological conditions, a reduced cellular proliferative capacity would be expected to be correlated with the upregulation of this factor. However, *Myod* and *myogenin*-induced expression of *Cdkn1a* also inhibits cyclin-dependent kinase activity to enable myocyte terminal differentiation [62, 63]. Hence, mSOD1 satellite cells in presymptomatic mice may experiment a reduction in their proliferative capacity, as above discussed, and a disturbed ability to differentiate, as reflected in the *Cdkn1a* and *myogenin* downregulation and as previously suggested in ALS patients [30].

Muscle type susceptibility to mSOD1 toxicity varies [44, 45, 64], and distinct regenerative response has been shown in fast- and slow-twitch myofibers from electrically stimulated [65], denervated and tetrodotoxin-treated muscles [66] and in SOD1-G93A mice [32]. One could, therefore, expect that myogenic cell cultures obtained from fast and slow myofibers of SOD1-G93A mice present differences at the functional and molecular levels. However, compared to the age-matched controls, mSOD1 SMSCs obtained from both fast and slow muscles displayed similar, although pathological state-dependent pattern of proliferation and MRF and *Cdkn1a* expression during the presymptomatic phases, when no severe denerv-

vation has been described. Because mSOD1 in the used model is expressed at similar levels in slow and fast muscles [32, 67], muscle type susceptibility to mSOD1 toxicity is probably directly linked to the distinct characteristics of motor neurons innervating type I and type II myofibers.

On the other hand, the observed impaired proliferative ability of mSOD1 satellite cells prompted us to decipher if myogenic differentiation was also disturbed in these cells. Although this was not the main purpose of the present study, myotube formation was observed in satellite cell cultures derived from SOD1-G93A mice at all the studied stages (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000338061). Moreover, previous studies performed in our group, maintaining the mSOD1 satellite cells in culture for 10 days demonstrated the formation of fully developed myotubes that occasionally contracted (see a video in the supplementary material of Manzano et al. [32]). However, the significant downregulation of *Cdkn1a* and *myogenin*, factors involved in differentiation induction [36], during the presymptomatic stages of the disease in mSOD1 cultures, indicates, at least, an abnormal induction of the myogenic program at the molecular level. Further studies will be necessary to confirm if the observed myotubes are fully functional or present the characteristics of wild-type cells.

To conclude, our data are consistent with the view that SMSC performance in both fast and slow muscles of the

SOD1-G93A muscle is disturbed. This is likely to be a cell-autonomous consequence of mSOD1 toxicity and not solely dependent on neuromuscular junction destruction or muscle niche input. In contrast, it is likely that the preferential fast muscle susceptibility in ALS mainly arises from signals at the cellular niche in vivo and from characteristics of innervating motor neurons, and not from differential effects of mSOD1 in these two muscle fiber types. To our knowledge, this is the first study demonstrating in vitro proliferation of SMSCs in a widely used mouse ALS model. Further studies are warranted to decipher which cellular mechanisms contribute to the impaired satellite cell proliferation and to further test whether these results apply to SOD1-G93A from very young animals or if prolonged postnatal time is required for mSOD1 toxicity. In conjunction, we consider our novel results of significant value to guide future research on muscle-targeted therapies for ALS.

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