TESIS DE LA UNIVERSIDAD DE ZARAGOZA

2011

9

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Caracterización de las células madre satélite musculares en un modelo murino de Esclerosis Lateral Amiotrófica

Departamento

Anatomía, Embriología y Genética Animal

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ISSN 2254-7606

Prensas de la Universidad Universidad Zaragoza

Universidad Zaragoza
1542

Tesis Doctoral

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Anatomía, Embriología y Genética Animal

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Instituto Aragonés de Ciencias de la Salud

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MEMORIA PRESENTADA POR:
Raquel Manzano Martínez

PARA OPTAR AL GRADO DE DOCTORA POR:
Universidad de Zaragoza

Septiembre, 2011

UNIVERSIDAD DE ZARAGOZA, 2011

LABORATORIO DE GENÉTICA BIOQUÍMICA (LAGENBIO)
Dpto. Anatomía, Embriología y Genética Animal
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UNIVERSIDAD DE ZARAGOZA / INSTITUTO ARAGONÉS DE CIENCIAS DE LA SALUD (IACS)

Caracterización de las células madre satélite musculares en un modelo murino de esclerosis lateral amiotrófica

Memoria presentada por

Raquel Manzano Martínez

Para optar al grado de Doctora por la Universidad de Zaragoza
Zaragoza, Septiembre 2011

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Doña **Rosario Osta Pinzolas**, Profesora Titular del Departamento de Anatomía, Embriología y Genética Animal de la Facultad de Veterinaria de la Universidad de Zaragoza,

CERTIFICA:

Que la memoria de Tesis Doctoral presentada por la licenciada doña **Raquel Manzano Martínez** con el título *"Caracterización de las células madre satélite musculares en un modelo murino de esclerosis lateral amiotrófica"* ha sido realizada bajo su dirección en el Departamento de Anatomía, Embriología y Genética Animal de la Facultad de Veterinaria de la Universidad de Zaragoza, y se corresponde con el Proyecto de Tesis aprobado por la Comisión de Doctorado en 2011, por lo que autoriza su presentación en la modalidad de compendio de publicaciones y con la Mención "Doctor Europeo" cumpliendo por lo tanto las condiciones requeridas para que su autora pueda optar al grado de Doctora por la Universidad de Zaragoza.

Y para que así conste, firma la presente en Zaragoza, a 16 de Junio de 2011.

Fdo.: Rosario Osta Pinzolas

A mi familia

La verdadera ciencia enseña, sobre todo, a dudar y a ser ignorante

Miguel de Unamuno

AGRADECIMIENTOS

AGRADECIMIENTOS

Ahora que ha llegado el momento de escribir este capítulo de agradecimientos me doy cuenta de que probablemente sean las líneas que más me ha costado redactar. No sólo porque es una de las partes más leídas de la tesis, sino porque me resulta casi imposible expresar en unas pocas palabras mi agradecimiento a tantas personas, que han contribuido de una u otra forma en este trabajo y porque, inevitablemente, seguro me olvidaré de mencionar a muchas otras.

En primer lugar me gustaría dar las gracias sinceramente a mi directora de tesis, Rosario Osta, su ayuda y sus consejos a lo largo de este tiempo, que han conseguido llevar a buen puerto este proyecto. Su motivación y entrega han sido un ejemplo para mí en esta carrera de fondo que es una tesis doctoral.

Me gustaría también mencionar a Pilar Zaragoza, por darme la oportunidad de entrar a formar parte de este grupo Lagenbio e iniciarme en investigación.

A Clemen e Inma, que con su permanente disposición para debatir, colaborar y ayudar en lo que ha estado en su mano han enriquecido de forma muy notable esta tesis.

A don Isaías, un ejemplo de vitalidad e ilusión que todos envidiamos.

A mis compañeros del grupo de investigación en ELA, los que están presentes, Ana Cris, Sara, Amaya y Samanta; y los que han pasado por él durante este tiempo, María, David, Janne, Marta... por vuestra inestimable ayuda en cada paso de esta tesis, poniendo y quitando PCRs, transportando ratones, extrayendo células o simplemente escuchando cuando parece que las cosas no van a salir nunca, os quiero dar las gracias especialmente.

No me olvido del resto de compañeros de laboratorio que he tenido durante estos años, Laura Ordovás, Laura Bericat, Salvador, Carmina, Jaber, Rosa, Fernando, Arianne, Carmen, Ana y Bea, y más recientemente Ana Rosa, Paola, Samuel y Diego. Por esos buenos ratos en la máquina de café, en las cenas, y sobre todo en el día a día; me habéis ayudado en todo lo que ha estado en vuestra mano, habéis contestado mis miles de preguntas, soportado a mis ratoncicos y sois los culpables de que llegue cada mañana con una sonrisa al laboratorio. Muchas gracias a todos.

Al departamento de Farmacología y Fisiología de la Facultad de Veterinaria de Zaragoza, por facilitarme sus equipos e instalaciones y, en especial a María Jesús Muñoz y Javi Miana por su ayuda, y por transmitir siempre una alegría, una fuerza y un optimismo envidiables y a José Ignacio por "cazar" mis ratones cuando era necesario.

A la gente de la UMI, en especial a Silvia, Pilar y a Jesús Navarro, por cuidar de mis ratoncitos y estar siempre pendientes de mis llamadas y de mis peticiones de transporte. Muchas gracias.

To the Molecular Genetics of Development Unit from Pasteur Institut in Paris. To Margaret Buckingham, Barbara Gayraud and specially to Mr. Didier Montarras and Giorgia Pallafacchina for letting me know most of the techniques used in this work, for answering my mails and my calls, for your advises, and specially for making my stay in Paris a great experience.

A la Dra. Ana Pérez, del CIMA en Pamplona, gracias por tus protocolos, por los anticuerpos, por tus consejos, por contestar a mis preguntas y en especial por haberme revelado esos pequeños detalles que casi siempre "marcan la diferencia".

A las Unidades de Microscopía e Imagen y de Anatomía Patológica del Instituto Aragonés de Ciencias de la Salud, en especial a María, Mamen y Alba, gracias por enseñarme a manejar microscopios, aconsejarme con mis células, por sus inmunos y sobre todo por entender mis prisas y hacerme un "huequito" en sus agendas.

Al Departamento de Parasitología de la Facultad de Veterinaria de Zaragoza, al Dr. Juan Antonio Castillo, Ana, Josemi, Julieta, Sara... y a Fanny del INA, por permitirme invadir vuestro espacio e instalar mis placas, mis medios, mis pipetas. Por aguantar el olor ratonil y por recibirme con una sonrisa. Gracias a todos.

A mis amigas Tania, Esther, Ana y Carol y a Carmen por los buenos ratos en el gimnasio, en las comidas, en las acampadas, en las fiestas, en los viajes, en los conciertos, en las granjas... y sobre todo por estar siempre ahí, por permitirme desconectar, por animarme, entenderme y saber apoyarme en todo este tiempo, MUCHAS GRACIAS!!!!

Finalmente quiero dar las gracias a mi familia. A mis padres que me han ayudado, aconsejado y "sufrido" en estos años. Vosotros habéis sido para mí un ejemplo constante de sacrificio, tenacidad y superación, sabed que sois los culpables de que haya terminado con éxito este proyecto y me habéis dado las fuerzas que me faltaban en algunos momentos.

A la *"tati-Lelawala"*, por tu alegría, tu optimismo y tu fuerza. Un párrafo es muy poco para agradecerte tu ayuda. SARA MANZANO, una parte muy importante de esta tesis es tuya.

A mis abuelos y tíos, por estar siempre ahí con una sonrisa, muchas gracias.

A Naxo, por comprender mis ausencias en muchas ocasiones, mis horarios difíciles, por tus consejos, por tu paciencia y por ser siempre un apoyo para mí.

Espero no olvidarme de nadie y si es así que se sienta representado en estas líneas. Gracias a todos de corazón, y espero seguir contando con vuestra ayuda y vuestra amistad siempre.

Este trabajo ha sido financiado a través de una ayuda para la elaboración de la Tesis Doctoral y obtención del título de Doctor concedida por el Gobierno de Aragón, y de los siguientes organismos y proyectos:

- *"Genética contra las enfermedades del sistema nervioso". Curar con genes enfermedades del Sistema Nervioso Central".* Financiado con el programa"Tú eliges Tú decides" de Caja Navarra. Año: 2008 y 2010.
- *"Caracterización de células Madre Adultas en un modelo animal de Esclerosis Lateral Amiotrófica y su relación con el desarrollo de la enfermedad".* Financiado con el programa PAMER del Instituto Aragonés de Ciencias de la Salud. Año 2008.
- *"Esclerosis Lateral Amiotrófica, ¿Neurodegeneración o falta de regeneración?".* Financiado con el programa PAMER del Instituto Aragonés de Ciencias de la Salud. Año 2009.
- *"Comportamiento de células madre en esclerosis lateral amiotrófica: satélites musculares, mesenquimales e iPS".* Financiado con el programa PAMER del Instituto Aragonés de Ciencias de la Salud. Año 2010.
- *"Estudio de genómica funcional en músculo esquelético en ratones modelo de esclerosis lateral amiotrófica y su modificación tras la aplicación de factores neurotróficos".* Financiado con el proyecto del Instituto de Salud Carlos III. Fondo de Investigación Sanitaria. PI071133. 2008-2011.
- *"Células madre como biomarcador y/o diana terapéutica en Esclerosis Lateral Amiotrófica".* Financiado con el proyecto del Instituto de Salud Carlos III. Fondo de Investigación Sanitaria. PI101787. 2011-2013.
- *Beca para estancia en centros nacionales y extranjeros de reconocido prestigio en el campo de la Medicina Regenerativa.* Finaciado con el Programa Aragonés de Medicina Regenerativa (PAMER) del Instituto Aragonés de Ciencias de la Salud. Año 2007.

La presente Tesis Doctoral está constituida por un compendio de trabajos de investigación previamente publicados y/o en proceso de publicación en diversas revistas científicas de carácter internacional.

Para ello, se presentan a continuación las referencias bibliográficas de cada uno de los artículos mencionados anteriormente:

1. Altered Expression of Myogenic Regulatory Factors in the Mouse Model of Amyotrophic Lateral Sclerosis. Raquel Manzano, Janne M. Toivonen, Sara Oliván, Ana C. Calvo, María Moreno-Igoa, María J. Muñoz, Pilar Zaragoza, Alberto García-Redondo y Rosario Osta. ***Neurodegenerative Diseases***. 2011;8(5):386-96. PMID: 21346327

2. Quantity and Activation of Myofiber-Associated Satellite Cells in a Mouse Model of Amyotrophic Lateral Sclerosis. Raquel Manzano, Janne M. Toivonen, Ana C. Calvo, Sara Oliván, Pilar Zaragoza, Maria Jesús Muñoz, Didier Montarras and Rosario Osta. ***Stem Cell Reviews and Reports***. 2011; DOI: 10.1007/s12015-011-9268-0. PMID:21537993

3. Sex, Fiber-Type And Age Dependent In Vitro Proliferation Of Mouse Muscle Satellite Cells. Raquel Manzano, Janne M. Toivonen, Ana C. Calvo, Francisco Javier Miana-Mena, Pilar Zaragoza, María Jesús Muñoz, Didier Montarras and Rosario Osta. ***Journal of Cellular Biochemistry***. 2011; 9999:1–13. PMID:21608019

4. Housekeeping gene expression in myogenic cell cultures from neurodegeneration and denervation animal models. Raquel Manzano, Janne M. Toivonen, Ana C. Calvo, María Jesús Muñoz, Pilar Zaragoza and Rosario Osta. ***Biochemical and Biophysical Research Communications***. 2011; 22;407(4):758-63. PMID:21439935

5. Altered in vitro proliferation of mouse SOD1-G93A skeletal muscle satellite cells. Raquel Manzano, Janne M. Toivonen, Ana C. Calvo, Sara Oliván, Pilar Zaragoza, Clementina Rodellar, Didier Montarras and Rosario Osta. ***Neurodegenerative Diseases*** (en revisión).

6. Impaired myogenic program in newborns of a mouse model of amyotrophic lateral sclerosis. Raquel Manzano, Janne M. Toivonen, Ana C. Calvo, Sara Oliván, Pilar Zaragoza, Clementina Rodellar, Didier Montarras and Rosario Osta. ***Journal of Cellular and Molecular Medicine*** (en revisión).

Los cuatro primeros manuscritos están publicados e indexados en revistas cuyo índice está incluído en el Journal of Citation Reports. Los manuscritos 2 y 3 se encuentran en proceso de edición final y aún no están disponibles en la base de datos MEDLINE, por lo que se adjunta el e-mail de aceptación de ambos así como sus números de referencia (PMID).

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RESUMEN

1. RESUMEN

La Esclerosis Lateral Amiotrófica (ELA), descrita por primera vez por Charcot en 1874, es una enfermedad neurodegenerativa caracterizada por la pérdida de neuronas motoras superiores e inferiores que cursa con atrofia y parálisis muscular progresiva. Aunque históricamente se ha considerado una enfermedad cuyos mecanismos etiopatogénicos se circunscriben a las motoneuronas, recientes trabajos ponen en entredicho estas afirmaciones y plantean la ELA como una afección multisistémica con implicación de mecanismos dentro y fuera del sistema nervioso. En concreto, y debido a su estrecha relación con el mantenimiento y supervivencia de las neuronas motoras, el músculo esquelético se ha revelado como un elemento esencial en el desarrollo de la enfermedad y como una interesante diana terapéutica.

En esta Tesis Doctoral se ha realizado un estudio de la capacidad de respuesta del músculo esquelético y en concreto de sus células madre satélite en el modelo murino de ELA SOD1-G93A. Para ello se ha determinado tanto el número, como la capacidad proliferativa y expresión de factores de regulación miogénica de estas células, *in vivo* e *in vitro,* en las distintas fases de la enfermedad y considerando músculos compuestos por distintos tipos de miofibras.

En primer lugar se estudió la evolución en la expresión de marcadores de daño muscular (*Rrad*) y denervación (*Chrna1*), así como del marcador de células satélite (*Pax7*) y los factores de regulación miogénica en el músculo esquelético en distintas fases de la enfermedad mediante técnicas de PCR en tiempo real y Western blot, comparándola con sus controles y animales axotomizados. Los resultados mostraron un incremento en *Pax7* y los factores de regulación miogénica paralelo al del marcador de denervación y más tardío que el marcador de daño muscular. Posteriormente se cuantificó, mediante inmunohistoquímica, el número y estado de activación de las células satélite en miofibras del músculo esquelético de animales SOD1-G93A a distintas edades y músculos según su composición en fibras de contracción rápida "fast" (extensor digital largo, EDL), o de contracción lenta "slow" (sóleo, SOL). Los resultados obtenidos indican que existe un menor número de células satélite musculares en animales transgénicos desde las fases presintomáticas tempranas de las enfermedad (40 días). Posteriormente, se observa una variación en estos parámetros que depende de la fase de la enfermedad y del tipo de fibras musculares. Por otra parte se validó el uso de seis genes normalizadores "housekeeping" en estudios de expresión génica de las células satélite murinas y se comprobó la

necesidad del uso de controles no transgénicos del mismo sexo, edad y tipo muscular en los ensayos *in vitro* con células satélite de modelos murinos de ELA. Finalmente se cuantificó la capacidad de proliferación y expresión de factores de regulación miogénica de dichas células SOD1-G93A mediante cultivos celulares en animales neonatos y en distintas fases de la enfermedad. En estos ensayos se observó una menor capacidad de proliferación de las células de los animales modelo respecto a sus controles, así como una modificación de dichos factores.

La alteración mostrada en el número y comportamiento de las células satélite musculares en el modelo animal SOD1-G93A desde estadios neonatales hasta estadios terminales, tanto *in vivo* como *in vitro,* se presenta como una futura diana terapéutica para la esclerosis lateral amiotrófica.

SUMMARY

Amyotrophic Lateral Sclerosis (ALS), firstly described by Charcot in 1874, is a neurodegenerative disease characterized by the loss of upper and lower motor neurons, which main hallmark is the atrophy and progressive muscle paralysis.

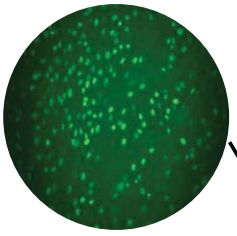
Although historically, it has been considered as a self-autonomous motor neuron disease, recent studies have challenged this theory and propose ALS as a multisystemic disease with multiple tissues implicated. Specifically, and due to its close relationship with the maintenance and survival of motor neurons, skeletal muscle is considered an essential element for the development of the disease and an interesting therapeutic target.

The aim of this Doctoral Thesis was the study of the skeletal muscle regenerative response capacity in the SOD1-G93A mouse model of ALS with special reference to its tissue committed stem cells, called "*satellite cells*". For this purpose the number and proliferative potential of these cells, as well as the myogenic regulatory factor expression *in vivo* and *in vitro,* was determined along the disease, considering muscles composed of different types of myofibers.

Firstly, the study of the muscle damage (*Rrad*) and denervation marker (*Chrna1*) gene expression was carried out as well as quantification of satellite cell marker (*Pax7*) and myogenic regulatory factors in skeletal muscle of SOD1-G93A mice at different stages of the disease by real time PCR and Western blot techniques; and results were compared to their wildtype littermate controls and axotomiced muscles. Results showed an increment in *Pax7* and myogenic regulatory factors

in parallel to the denervation marker, and latter than that observed in the muscle damage marker. Subsequently, immunohistochemistry was performed to quantify the number and activation status of satellite cells in myofibers from different SOD1-G93A skeletal muscles and different stages of the disease. Results demonstrated a diminished number of satellite cells in myofibers from transgenic mice from the early pre-symptomatic stages of the disease. Subsequently, the variation of these parameters depended on the disease stage and skeletal muscle fiber-type. In the other hand, the use of six candidate housekeeping genes was validated in gene expression studies of mouse skeletal muscle satellite cells and the need to include age, sex and muscle type matched wildtype controls in *in vitro* assays with murine ALS model satellite cells was probed. Finally, the proliferation rate and the myogenic regulatory factor expression were quantified in these SOD1-G93A cells in cell cultures from neonatal to terminal stages. A reduced proliferative capacity of the cells and the modification of these factors were observed.

The disturbance of the number and behaviour of the skeletal muscle satellite cells in the animal model SOD1-G93A from neonatal to terminal stages *in vivo* and *in vitro* represents a promising therapeutic target in amyotrophic lateral sclerosis.



INTRODUCCIÓN

2. INTRODUCCIÓN

2.1. Antecedentes

Las enfermedades neurodegenerativas (Parkinson, Alzheimer, Esclerosis Lateral Amiotrófica o Atrofia Muscular Espinal) son un gran reto para la medicina del sXXI debido al envejecimiento de la población. La esclerosis lateral amiotrófica (ELA), enfermedad con la que se va a trabajar en este estudio, a pesar de considerarse una enfermedad rara, es la cuarta enfermedad neurodegenerativa y afecta a unas 4000 personas en nuestro país. Actualmente existen varias teorías que explican la neurodegeneración pero muy pocas tratan de manera específica por qué se produce la muerte selectiva de las neuronas motoras en la ELA. Los mecanismos etiopatogénicos más extendidos son la excitotoxicidad, el daño oxidativo, la alteración en la homeostasis del calcio, el mal plegamiento proteínico, el entorpecimiento del transporte axonal, la alteración de la producción de energía y la apoptosis. En los últimos años además, la implicación de la glía, y más recientemente, también del músculo, se han convertido en una parte básica para explicar la degeneración de las motoneuronas. La interacción alterada entre el músculo y la neurona motora podría conducir a la muerte de ésta última en el caso de la ELA. De esta forma, se está pasando de la creencia inicial de que la muerte de las motoneuronas era causada exclusivamente por un daño directo a éstas, a la aceptación de que la ELA podría ser una enfermedad multisistémica en la que estarían implicados muchos tipos celulares (músculo, glía, astrocitos...). Finalmente se ha sugerido que la neurodegeneración podría agravarse por una falta de regeneración, es decir, que la reparación defectuosa de los tejidos por las células madre adultas alteradas empeora la patología. En este sentido, es posible plantear que la ELA sea una enfermedad multisistémica en la cual exista una alteración en parámetros fisiológicos, estructurales y metabólicos en distintos tipos celulares (músculo, glía, neurona motora...). Todo esto lleva a considerar tejidos no neuronales como el músculo esquelético, como dianas terapéuticas para la ELA más accesibles que el sistema nervioso.

El trabajo presentado para optar al grado de Doctora pretende determinar si existe alteración en la capacidad de regeneración muscular en la enfermedad. Para ello se han caracterizado los factores de regulación miogénica y la proliferación de las células madre musculares (células satélite) en el modelo animal de esclerosis lateral amiotrófica SOD1-G93A. En el siguiente apartado se realizará una breve revisión

de la enfermedad y de la regeneración muscular, los objetivos planteados y un resumen de la metodología utilizada y de los resultados obtenidos que se encuentran ampliamente descritos en las publicaciones presentadas.

2.2. Presentación de los trabajos y justificación de su unidad temática

2.2.1. Esclerosis Lateral Amiotrófica (ELA)

La esclerosis lateral amiotrófica (ELA) se define como un desorden neurodegenerativo caracterizado por una pérdida de las motoneuronas superiores e inferiores localizadas en la corteza motora, el tronco del encéfalo y la médula espinal. También se conoce como enfermedad de Lou Gehrig desde que este jugador de fútbol de los Yankees la padeciera en 1941. Etimológicamente, la palabra "amiotrófica" refleja la atrofia muscular que genera debilidad muscular y fasciculaciones; y "esclerosis lateral" se refiere al endurecimiento de las astas anteriores y laterales de los tractos corticoespinales por la degeneración de las motoneuronas localizadas en estas áreas y la gliosis (Rowland, L.P. y cols. 2001). Epidemiológicamente la ELA se considera como una enfermedad rara ya que afecta aproximadamente a un 0,05% de la población. Cada año se diagnostican 900 nuevos casos de ELA en España, lo que representa un número total de casos de 4000. La incidencia de esta enfermedad es de 2 casos por cada 100.000 habitantes al año y su prevalencia es de 1 por cada 10.000. Por lo tanto, 40.000 españoles desarrollarán la enfermedad a lo largo de su vida (www.fundela.info). Estos datos son uniformes en toda la Unión Europea y América. En función de su presentación clínica, la ELA puede dividirse en dos formas. En la "forma clásica o espinal" los primeros síntomas son fasciculaciones, calambres, espasticidad e hipereflexia como consecuencia de la disfunción de las motoneuronas superiores y debilidad, atrofia muscular y parálisis derivados de la alteración de las motoneuronas inferiores. La otra presentación de la enfermedad es en "forma bulbar", que inicialmente produce disartria en el lenguaje, disfagia, sialorrea y debilidad facial bilateral que afecta a la parte inferior de la cara. En un número significativo de casos los síntomas incluyen también labilidad emocional y bostezo excesivo. Finalmente, la forma bulbar evoluciona a forma espinal (Wijesekera, L.C. y cols. 2009). Ambas formas se caracterizan además por la preservación de los músculos que controlan el movimiento de los ojos y los esfínteres urinarios (McGuire, V. y cols. 1996) (Hayashi, H. y cols. 1989; Sasaki, S. y cols. 1992). El inicio de la enfer-

medad depende de su etiología y habitualmente la muerte sobreviene por un fallo en los músculos de la respiración de 1 a 5 años tras el inicio de los síntomas (Rowland, L.P. 1998). Debido a la variabilidad de sus síntomas, el diagnóstico de la enfermedad no es sencillo. El primer criterio diagnóstico consensuado fue definido por un subcomité de la Federación Mundial de Neurología (WFN) para la ELA, en una reunión celebrada en España en la que se plantearon unas directrices internacionales comunes, son los denominados "Criterios de El Escorial", que fueron publicados en 1994 y revisados en 2000 (www.wfneurology.org; Brooks, B.R. y cols. 2000). En estos criterios se establece que para el diagnóstico de ELA es necesaria la presencia de signos clínicos de alteración de la motoneurona superior en al menos una de las regiones bulbar, cervical o lumbosacra, así como un desarrollo crónico y progresivo. Además es necesario el hallazgo de alteraciones de la motoneurona inferior en al menos dos extremidades mediante el análisis clínico, neuropatológico o electrofisiológico. Debe existir una ausencia de signos sensoriales, anormalidades neurogénicas de los esfínteres y alteraciones clínicamente evidentes del sistema nervioso central o periférico que pudiesen explicar los signos clínicos y electrofisiológicos. Por último, se acuerda que el diagnóstico probado de ELA únicamente puede realizarse mediante histología *post mortem*. En la actualidad, además del estudio del historial clínico del paciente, entre las técnicas reconocidas para el diagnóstico de esta enfermedad se encuentran las técnicas de neuroimagen, inmunohistoquímica, análisis genómico, presencia en electromiografía de fasciculaciones o señales de daño neuronal, técnicas de estimulación magnética transcraneal, morfometría voxel, biopsias musculares y estudios neuropatológicos. En estos momentos, el diagnóstico de ELA se clasifica también en función del número de zonas afectadas en las categorías de "clínicamente posible", "clínicamente probable", "clínicamente probable probado laboratorialmente" y "clínicamente definida". (Figura 1.1) (Brooks, B.R. y cols. 2000; de Carvalho, M. y cols. 2008; Wijesekera, L.C. y cols. 2009).

El diagnóstico *post mortem* está basado en alteraciones histopatológicas. En este sentido, las observaciones características son la degeneración y pérdida de motoneuronas con gliosis astrocítica y presencia de inclusiones intraneuronales en neuronas en degeneración y glía (Ince, P.G. y cols. 2003; Ince, P.G. y cols. 2007). Dichas inclusiones y su naturaleza constituyen un elemento altamente específico de la enfermedad y por lo tanto una herramienta clave en el diagnóstico *post mortem* de esta patología. Son de tres tipos: Cuerpos de Bunina (Bunina, T.L. 1962; Piao, Y.S. y cols. 2003; Mizuno, Y. y cols. 2006; Okamoto, K. y cols. 2008), inclusiones

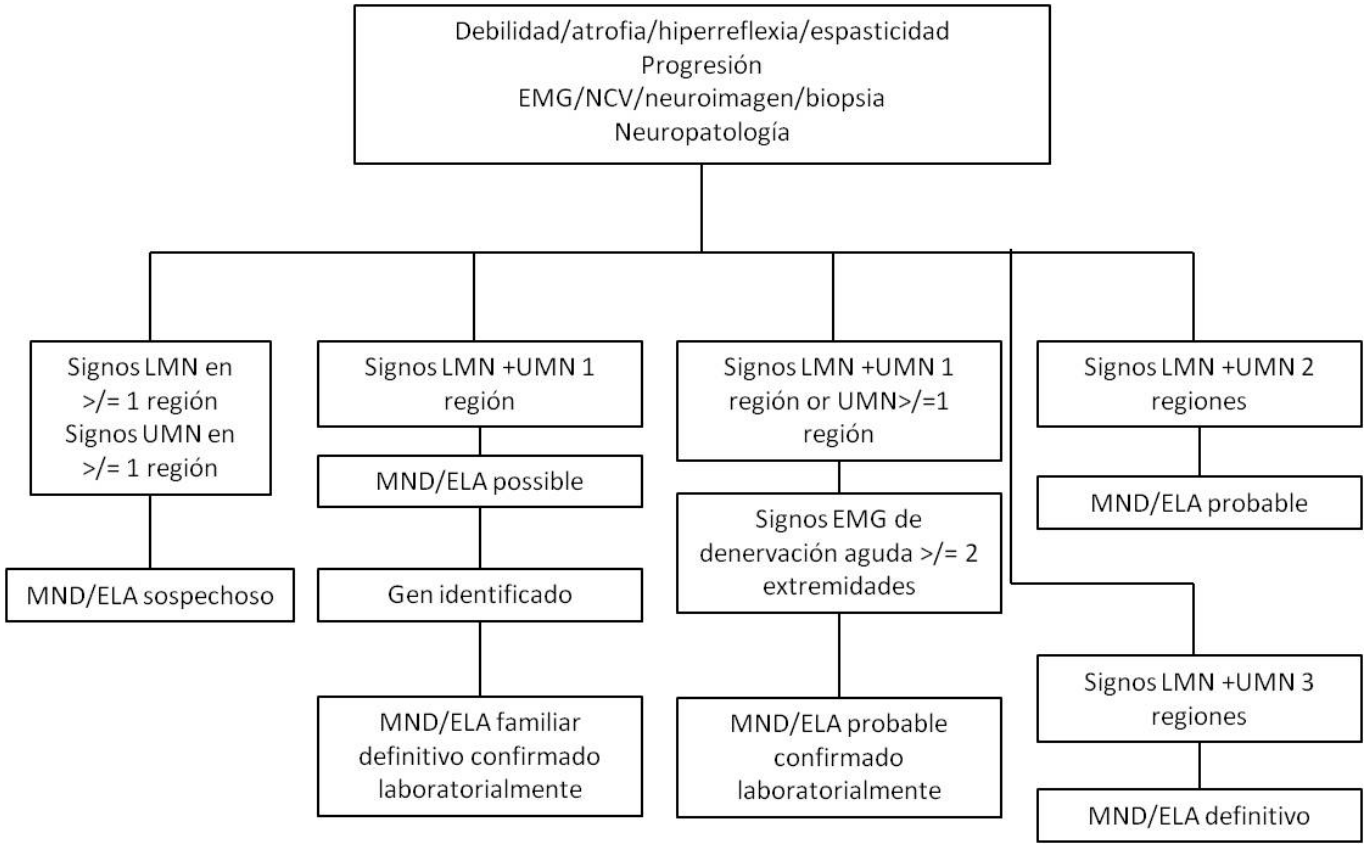


Figure 2.1: *Esquema de los criterios revisados de El Escorial para el diagnóstico de ELA. EMG: electromiografía; NCV: test de velocidad de la conducción nerviosa; LMN: motoneurona inferior; UMN: motoneurona superior; MND: enfermedad de la motoneurona; (modificado de Mitchell, J.D. 2000).*

ubiquitinadas o inmunoreactivas para ubiquitina (UBIs e Ub-IR); cuyo componente mayoritario es la proteína de unión a ADN TAR 43 (TDP-43) (Leigh, P.N. y cols. 1991; Ince, P.G. y cols. 2003; Neumann, M. y cols. 2006; Tan, C.F. y cols. 2007; Liscic, R.M. y cols. 2008) e inclusiones de conglomerados hialinos de neurofilamentos (HCIs) (Leigh, P.N. y cols. 1989).

A pesar de los avances en las técnicas diagnósticas, así como en el consenso de los criterios diagnósticos de la enfermedad, se calcula que existe entre un 9 y un 15% de falsos positivos. El diagnóstico diferencial de la ELA se centra principalmente en lesiones traumáticas, mielopatía espondilótica cervical, otras mielopatías cervicales como lesiones del agujero magno, tumores intrínsecos y extrínsecos, siringomielia, miositis por cuerpos de inclusión (IBM), neuropatía motora multifocal (MFMN) y enfermedad de Kennedy, otras enfermedades neurodegenerativas como Parkinson y alteraciones de las uniones neuromusculares como la miastenia gravis (MG) (Wijesekera, L.C. y cols. 2009). A pesar de que su diagnóstico es principalmente clínico, los hallazgos histopatológicos han permitido el desarrollo de diversas teorías acerca de la etiología de esta enfermedad. La etiología de la ELA en la mayoría de los casos es desconocida. Sin embargo se han propuesto diversos factores que pueden estar implicados en mayor o menor modo en su desarrollo, como los factores genéticos (Wijesekera, L.C. y cols. 2009). Atendiendo a este criterio la mayoría de las clasificaciones hablan de la ELAF (esclerosis lateral amiotrófica familiar) donde se englobarían los casos con antecedentes familiares. Dentro de este tipo se clasifican las distintas variantes de ELA en función, bien del gen alterado o bien del tipo de herencia y constituyen apenas un 10% de los casos de ELA. La herencia en la mayor parte de los casos es de tipo autosómico dominante, aunque también se han descrito casos de herencia autosómica recesiva, maternal por mutaciones en locus del genoma mitocondrial (COX1 y IARS2) y una familia con una mutación que presenta herencia dominante ligada al cromosoma X (Siddique, T., y cols. 1998). El gen más comúnmente alterado en la ELA es el gen de la superóxido dismutasa 1 (SOD1) que se encuentra modificado en el 12-23% de los casos de ELAF (Andersen, P.M. 2006). Existen 149 mutaciones localizadas en la cadena polipeptídica de la SOD1 que se han relacionado con la enfermedad y que en su mayor parte generan sustituciones aminoacídicas puntuales (alsod.iop.kcl.ac.uk; Deng, H.X. y cols. 2008). A nivel proteínico, la enzima codificada por este gen se denomina superóxido dismutasa 1 soluble (SOD1). Es una metaloenzima antioxidante de 153 aminoácidos y 16KDa encargada de la protección del organismo frente al acúmulo de radicales libres como

el anión superóxido (O_2^-). Se trata de una enzima localizada constitutivamente en el citoplasma, núcleo, peroxisomas y espacio intermembrana mitocondrial de las células de mamífero (Crapo, J.D. y cols. 1992; Zelko, I.N. y cols. 2002). Funcionalmente se encarga de detoxificar especies reactivas de oxígeno (ROS), como el anión superóxido (O_2^-) o hidroxilo (OH^-), mediante oxidaciones y reducciones cíclicas del cobre de su sitio activo generando peróxido de hidrógeno (H_2O_2), que posteriormente será transformado por las enzimas glutatión peroxidasa o catalasa en agua (H_2O) y oxígeno molecular (O_2). Por otra parte, se encuentra la ELAE (esclerosis lateral amiotrófica esporádica), donde se engloban los casos donde no se conoce un historial familiar de afectados por la enfermedad. Este tipo corresponde al 85-90% de los casos, y aunque se han desarrollado numerosas teorías acerca de su origen no se ha descrito una relación clara y directa con ninguno de ellos. De este modo, se ha planteado la posibilidad de que la exposición a ciertos elementos ambientales como fertilizantes, pesticidas, herbicidas o metales pesados constituya un factor de riesgo de padecimiento de ELAE (Govoni, V. y cols. 2005; Weisskopf, M.G. y cols. 2009; Vinceti, M. y cols. 2010). Por otra parte, otros factores como eventos cerebrales traumáticos, consumo de tabaco, presencia del alelo APOE-4 o actividad física intensa también se han considerado como predisponentes al padecimiento de la enfermedad (Longstreth, W.T. y cols. 1998; Veldink, J.H. y cols. 2005; Chio, A. y cols. 2009; Schmidt, S. y cols. 2010). Algunos autores describen también una estrecha relación con agentes infecciosos víricos o bacterianos persistentes como retrovirus endógenos (HERV-K) o enterovirus (Woodall, C.J. y cols. 1994) y virus de la inmunodeficiencia humana (HIV) (Moulignier, A. y cols. 2001; Zoccolella, S. y cols. 2002; Goos, M. y cols. 2007). Finalmente la esclerosis lateral amiotrófica esporádica ha sido también en ciertos momentos catalogada como una enfermedad con origen autoinmune (Conradi, S. y cols. 1993; Niebroj-Dobosz, I. y cols. 1999) o un síndrome neurológico paraneoplásico, aunque en estudios posteriores estos descubrimientos no se confirmaron (Geen, J. y cols. 2000; Vigliani, M.C. y cols. 2000). Por último, se sospecha que en ciertos casos de ELA esporádica, existen mutaciones genéticas implicadas, conformando lo que se denomina ELA aparente. Así, en el 2-3% de los casos de ELAE también se han descrito mutaciones en el gen de la SOD1 (Andersen, P.M. 2006). Hasta la actualidad estudios genómicos de algunos casos de ELAE han revelado la presencia de anomalías en un número elevado de genes diferentes (Meyer, T. y cols. 2003). Sin embargo, hay que tomar estos datos con precaución, ya que cabe la posibilidad de que parte de estos casos sean en realidad

ELA familiar, en los que por diversas razones no se haya llegado a un diagnóstico de los familiares afectados. La media de edad en el inicio sintomático se sitúa entre 55 y 65 años en ELA esporádica (ELAE, casos sin historia de familiares afectados) y 10 años antes en ELA familiar (ELAF, casos con familiares afectados). Sólo un 5% de los casos presentan una "forma juvenil" con inicio antes de los 30 años (Wijesekera, L.C. y cols. 2009). A nivel epidemiológico, las diferencias entre la ELAF y la ELAE se centran básicamente en el ratio de hombres y mujeres afectados siendo 1:1 en ELAF y 1.5:1 en ELAE, aunque en este último caso el ratio tiende a igualarse con la edad; esto se ha asociado a factores hormonales protectores en las mujeres, así como a una mayor exposición a factores de riesgo de los hombres. Finalmente, el tiempo de supervivencia medio de los pacientes con ELA familiar es de 1.1 años mientras que en ELA esporádico es de 2.6 años (Li, T.M. y cols. 1988; Wijesekera, L.C. y cols. 2009). Considerando toda esta información, cada vez hay más hipótesis que apuntan a que la suma de distintos fenómenos de predisposición genética y exposición a factores de riesgo pueden conducir al desarrollo de la enfermedad.

A pesar de que desde su descubrimiento ha habido avances significativos para descifrar las causas de la enfermedad y sus mecanismos patogénicos, el desconocimiento del origen exacto de los mismos hace que su tratamiento sea muy complicado. En la actualidad, las terapias de las que se benefician los pacientes son básicamente paliativas a cargo de un equipo multidisciplinar que incluye neurólogos, fisioterapeutas, psicólogos, nutricionistas y terapeutas ocupacionales entre otros especialistas. A nivel de mecanismos patogénicos existe un único compuesto comercializado, denominado riluzol. Es un inhibidor de la liberación presináptica de glutamato y por lo tanto se cree que ejerce su acción contrarrestando la excitotoxicidad mediada por este aminoácido descrita en la ELA (Bensimon, G. y cols. 1994; Lacomblez, L. y cols. 1996). Sin embargo, los efectos de este fármaco son limitados ya que se ha demostrado que prolonga la vida de los pacientes solo en algunos casos y en 2 a 3 meses. A nivel experimental se han ensayado gran variedad de terapias para contrarrestar los distintos mecanismos observados en la patogenia de la enfermedad. Es el caso de tratamientos antiagregación protéica como el arimoclomol o la infusión de inmunoglobulinas (Kieran, D. y cols. 2004; Urushitani, M. y cols. 2007; Takeuchi, S. y cols. 2010), o que favorecen la función mitocondrial (Zhu, S. y cols. 2002; Shefner, J.M. y cols. 2004) y de factores de crecimiento (Zheng, C. y cols. 2004). También se han ensayado compuestos que tratan de disminuir los niveles de RNA mensajero del gen mutado mediante oligonucleótidos antisentido o pequeñas moléculas de RNA

inhibidoras (Smith, R.A. y cols. 2006; Wang, H. y cols. 2008). Nuestro grupo, ha desarrollado un tratamiento experimental mediante terapia génica no viral (DNA desnudo) basado en el efecto neuroprotector del fragmento C de la toxina tetánica (Moreno-Igoa, M. y cols. 2010). Dicho tratamiento está siendo estudiado por una Asociación de Interés Económico para ser trasladado a la clínica. Finalmente el uso de células madre se ha barajado como alternativa para el tratamiento de la ELA. Se ha demostrado que la estrategia con mayores beneficios se centra en la inoculación de células madre mesenquimales y de médula ósea directamente en médula espinal y corteza motora. El mecanismo protector de estas células sería a través de su diferenciación a células no neuronales capaces de proteger las motoneuronas supervivientes, de proveer de factores neurotróficos a éstas y de detoxificar el entorno (Clement, A.M. y cols. 2003; Mazzini, L. y cols. 2008; Deda, H. y cols. 2009; Martinez, H.R. y cols. 2009; Xu, L. y cols. 2009). De especial relevancia para este trabajo son los resultados preliminares de nuestro grupo y el Dr. Salvador Martínez donde se observa una mejoría en modelos animales de ELA tras es trasplante de células madre de médula ósea sanas mediante inoculación muscular. Este reciente trabajo resalta la eficacia de las terapias dirigidas a mejorar el entorno neuronal y no directamente las motoneuronas.

Por otra parte, el conocimiento de la fuerte implicación del músculo esquelético en el desarrollo de la enfermedad ha favorecido la aparición de compuestos y técnicas que tienen como diana este tejido. Es el caso de los inhibidores de la miostatina (Lee, S.J. y cols. 2005; Cadena, S.M. y cols. 2010) o activadores de la troponina (Shefner, J.M. y cols. 2004) y la expresión de factores de crecimiento como Igf-1 a nivel muscular (Musaro, A. y cols. 2001; Kaspar, B.K. y cols. 2003; Dobrowolny, G. y cols. 2005; Dobrowolny, G. y cols. 2008a). Estas técnicas abren la puerta a terapias musculares, menos invasivas pero igualmente eficaces en contrarrestar el avance de la enfermedad ya sea en solitario o combinadas con el tratamiento dirigidos a otros tejidos. Sin embargo, para la mejora de los planteamientos terapéuticos actuales resulta indispensable el conocimiento de los mecanismos patogénicos que desencadenan los procesos observados en la ELA. Existen varias hipótesis que pueden explicar la alteración y pérdida selectiva de las motoneuronas, característica principal de la ELA, aunque ninguna de ellas es concluyente. La primera de ellas habla de una toxicidad inducida por la propia enzima mutada. La mutación originaría una perturbación del metabolismo del oxígeno y una ganancia de función de la enzima al permitir el acceso a su centro activo de sustratos atípicos, y la inducción de re-

acciones aberrantes que generan radicales libres como el hidroxilo (OH^-), originando un estrés oxidativo (Beckman, J.S. y cols. 1993; Wiedau-Pazos, M. y cols. 1996; Estevez, A.G. y cols. 1999) o liberando el cobre y zinc neurotóxicos de su centro activo (Figura 1.2).

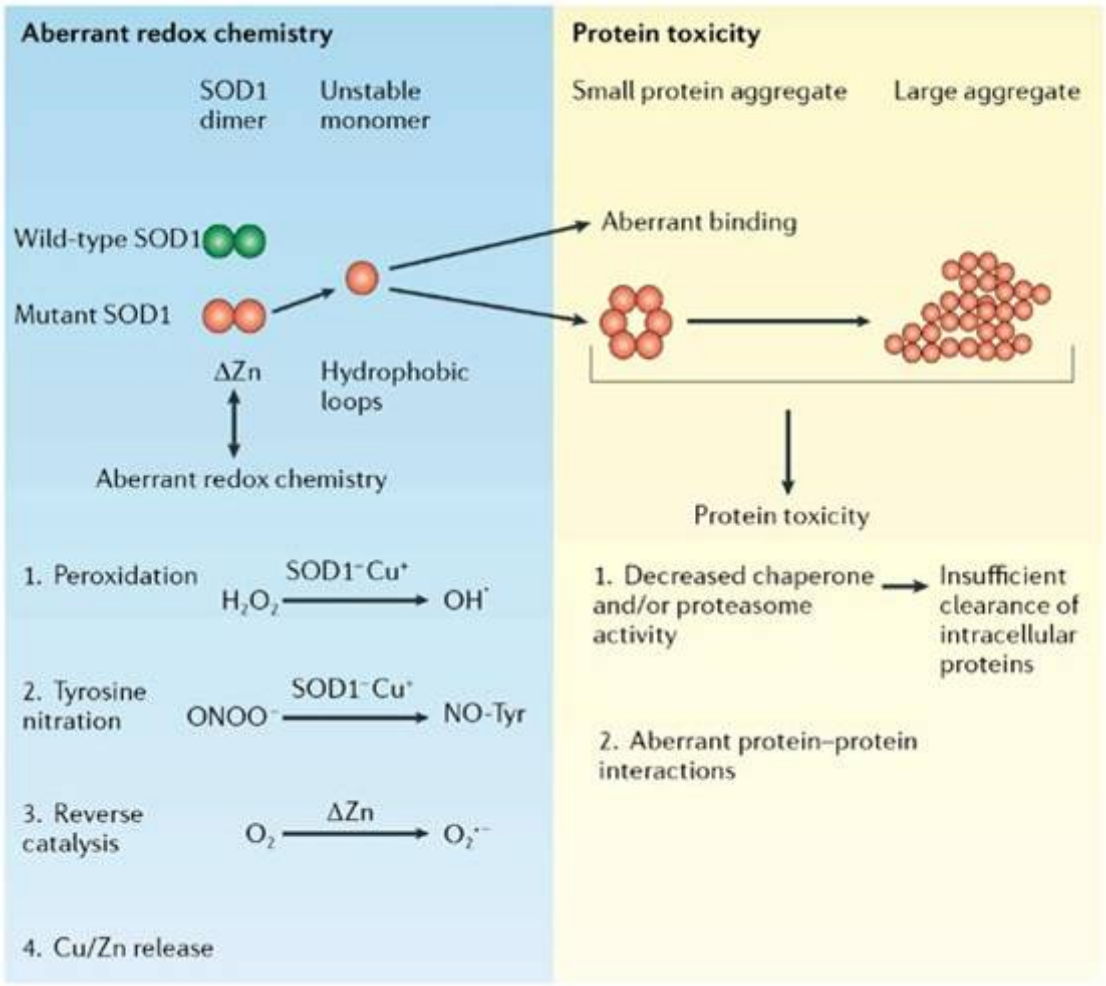


Figure 2.2: *Modelos de toxicidad mediada por la SOD1 mutada (Pasinelli, P. y cols. 2006).*

Por otro lado la mutación de la enzima podría originar una inestabilidad conformacional, reducción de su carga repulsiva neta y mal plegamiento lo que podría desencadenar su agregación (Stieber, A. y cols. 2000; Lindberg, M.J. y cols. 2005; Rousseau, F. y cols. 2006; Sandelin, E. y cols. 2007). La toxicidad de estos agregados podría deberse a su interferencia o saturación de la actividad del proteasoma, impidiendo la degradación y reciclaje normal tanto de la SOD1, como de otras pro-

teínas (Hoffman, E.K. y cols. 1996; Urushitani, M. y cols. 2002; Puttaparthi, K. y cols. 2003), la interacción e inhibición de la actividad de las chaperonas (Bruening, W. y cols. 1999; Shinder, G.A. y cols. 2001; Watanabe, M. y cols. 2001; Koyama, S. y cols. 2006) o el secuestro y alteración de la toxicidad de otras proteínas cruciales para procesos celulares como Bcl-2 (Pasinelli, P. y cols. 2004). Finalmente, también existe la posibilidad de que esta agregación sea un intento de disminuir los niveles de SOD1 mutada en contacto con la célula, y por lo tanto un mecanismo de protección (Witan, H. y cols. 2008; Witan, H. y cols. 2009) (Figura 1.2).

Otra de las hipótesis propuestas es la excitotoxicidad el glutamato. La excitotoxicidad es un proceso por el cual un incremento en los niveles de glutamato extracelular, o la alteración y sensibilización de la neurona postsináptica a este aminoácido, genera una excesiva estimulación de las células nerviosas produciendo daño y degeneración de las mismas. En condiciones normales, el glutamato es sintetizado y liberado por las neuronas presinápticas en vesículas al espacio extracelular, tras interaccionar con sus receptores de las membranas postsinápticas, es captado por transportadores específicos (EAAT14) localizados principalmente en los astrocitos. En éstos, es degradado a glutamina por la enzima glutamina sintetasa y puesto a disposición de las neuronas para volver a sintetizar glutamato. En pacientes y modelos de ELA se han encontrado evidencias de fallos en los mecanismos de limpieza del glutamato; se han descrito concentraciones elevadas de glutamato en plasma (Andreadou, E. y cols. 2008), alteraciones de la expresión de glutamina sintetasa (Bos, I.W. y cols. 2006) y disminución de la captación de esta molécula y de la expresión y actividad de sus transportadores astrogliales tipo EAAT2 (Rothstein, J.D. y cols. 1992; Ferrarese, C. y cols. 2001). Por otra parte, existen estudios que demuestran que la alteración de los receptores postsinápticos AMPA de este neurotransmisor, así como la sensibilización de las motoneuronas a ésta molécula, contribuyen también a la excitotoxicidad (Kwak, S. y cols. 2005). Por un lado, la presencia de altos niveles de glutamato extracelular sobre-estimula sus receptores NMDA, y la no modificación de sus receptores AMPA cambia su permeabilidad; ambos fenómenos favorecen la entrada masiva de calcio al citoplasma, saturando los orgánulos encargados de tamponarlo como el retículo endoplásmico y sobre todo las mitocondrias.

Como hemos visto en el apartado anterior uno de los componentes más vulnerables a la excitotoxicidad son las mitocondrias. En ellas recae principalmente la función de tamponar los niveles intracelulares de calcio. A nivel morfológico se

han observado modificaciones mitocondriales como agregados en regiones subsarcolémicas (Sasaki, S. y cols. 1996), mitocondrias con elongaciones de su membrana externa (Hirano, A. y cols. 1984) o "mitocondrias extrañas gigantes" con inclusiones paracristalinas (Nakano, Y. y cols. 1987). A nivel bioquímico, se han observado incrementos en la actividad de los complejos de las cadenas respiratorias mitocondriales (Bowling, A.C. y cols. 1993; Browne, S.E. y cols. 1998) o alteraciones en su genoma (Vielhaber, S. y cols. 2000). En estudios *in vitro*, en líneas neuronales que expresaban SOD1 mutada, se vio un incremento de calcio citosólico y una disminución en el potencial de acción de la membrana mitocondrial; hechos que se asociaban con una incapacidad de la mitocondria para secuestrar y tamponar los niveles de calcio citosólicos (Carri, M.T. y cols. 1997; Jaiswal, M.K. y cols. 2009). Sin embargo, en los pacientes de ELA, los niveles de calcio mitocondrial se encuentran incrementados lo que a priori desmontaría la hipótesis anterior (Siklos, L. y cols. 1996).

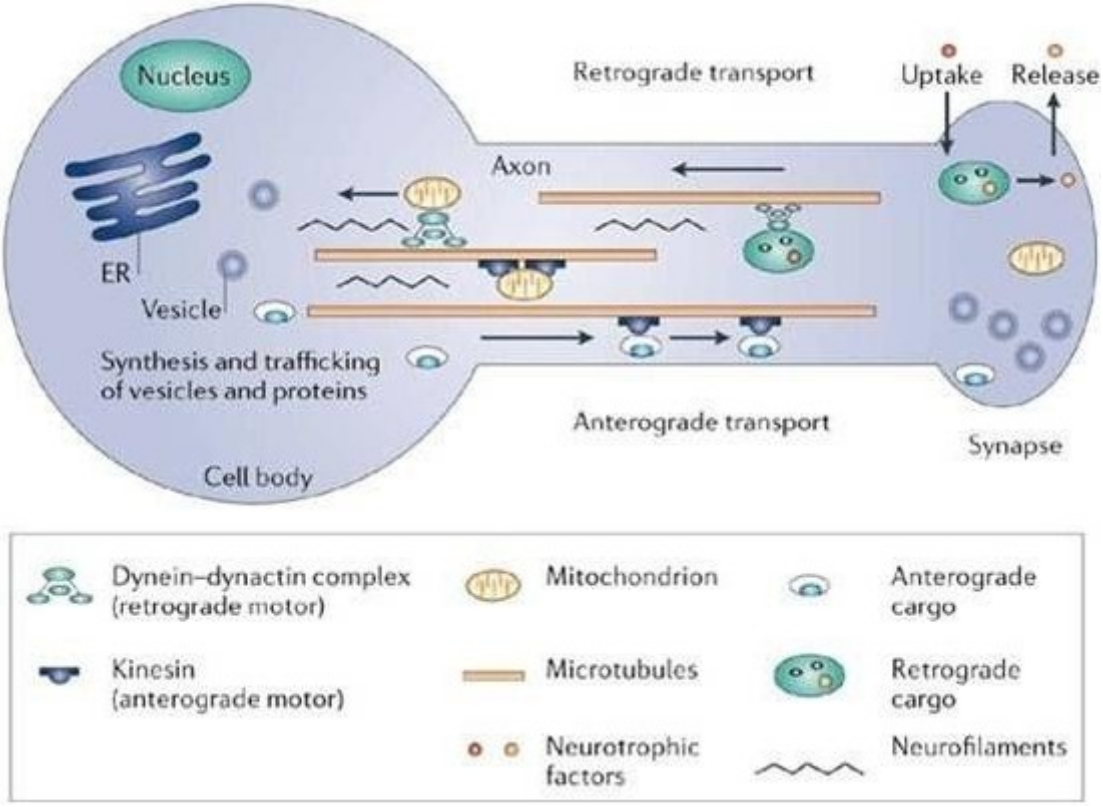


Figura 2.3: *Representación esquemática del mecanismo propuesto para el daño a las motoneuronas mediado por alteración del transporte axonal (Pasinelli, P. y cols. 2006).*

Por último, el transporte de moléculas y orgánulos es un proceso fundamental para el desarrollo, función y supervivencia especialmente en las motoneuronas ya que se encuentran entre las células más grandes y largas del organismo. Alteraciones en el transporte axonal podrían reflejarse en una pérdida del patrón arquitectónico del sistema de neurofilamentos (NFs) (Figura 1.3).

Se ha descrito la reducción de la actividad del transporte axonal en pacientes y en ratones modelo de ELA (Sasaki, S. y cols. 2005). También se han encontrado mutaciones en el gen de la subunidad pesada de los NFs en pacientes con ELA esporádica y familiar (Figlewicz, D.A. y cols. 1994; Al-Chalabi, A. y cols. 1999).

Además, los niveles de mRNA de la subunidad ligera de los NFs en los pacientes con ELA esporádica son anormalmente bajos (Bergeron, C. y cols. 1994). Se piensa que la expresión aberrante de los NFs podría impedir el transporte de moléculas necesarias para el mantenimiento de los axones, como una "estrangulación axonal" (Willard, M. y cols. 1983; Collard, J.F. y cols. 1995). Por otra parte, se sabe que el transporte axonal anterógrado de ratones transgénicos que sobreexpresan la enzima mutada humana SOD1-G93A y SOD1-G37R se encuentra decelerado antes del comienzo de la enfermedad y se agrava a medida que avanza ésta (Zhang, B. y cols. 1997; Borchelt, D.R. y cols. 1998; Williamson, T.L. y cols. 1999). Algunos autores sugieren que los agregados de NFs en los axones proximales (esferoides) podría comprometer físicamente el aparato de transporte, al menos en el tráfico anterógrado (Rao, M.V. y cols. 2003), aunque este punto no está del todo aclarado. Asimismo, el transporte retrógrado se ha visto afectado en ratones modelo de ELA (Murakami, T. y cols. 2001), y ha sido atribuido a la deslocalización y alteración de la función de la dineína (Ligon, L.A. y cols. 2005). Por lo tanto, independientemente del mecanismo, varios hallazgos indican que los defectos en el transporte axonal retrógrado y anterógrado podrían contribuir a la muerte de motoneuronas en la ELA.

A pesar de su compleja patogenia, parece claro que la característica principal de la enfermedad es una degeneración selectiva de las motoneuronas superiores e inferiores (Figura 1.4).

Sin embargo, cabría preguntarse, ¿es la ELA una enfermedad exclusiva de las motoneuronas?. A este respecto existen varios trabajos que corroboran que la patología observada en la ELA no deriva exclusivamente de un daño a las motoneuronas y existe una participación de células nerviosas no neuronales. En primer lugar, existen estudios que demuestran que la expresión restringida de SOD1 mutada en mo-

toneuronas (Pramatarova, A. y cols. 2001; Lino, M.M. y cols. 2002), astrocitos (Gong, Y.H. y cols. 2000; Beers, D.R. y cols. 2006) o microglía (Beers, D.R. y cols. 2006) no induce neurodegeneración. Además, la reducción de los niveles de esta enzima en motoneuronas mediante RNA de interferencia retrasó el inicio pero no ralentizó el progreso de la enfermedad (Miller, T.M. y cols. 2005; Ralph, G.S. y cols. 2005). Por otra parte en el co-cultivo de astrocitos o microglía que portan la enzima mutada con motoneuronas procedentes de animales sanos, se observa que células mutadas inducen neurotoxicidad hacia las sanas produciendo su muerte (Nagai, M. y cols. 2007; Xiao, Q. y cols. 2007). *In vivo,* la disminución o abolición de la expresión de SOD1 mutada en células de la microglía de ratones retrasó la progresión de la enfermedad e incrementó la supervivencia (Beers, D.R. y cols. 2006; Boillee, S. y cols. 2006). De acuerdo con estos resultados se observó además que la generación de ratones quimera con diferentes porcentajes de SOD1 mutada en motoneuronas, astrocitos o microglía, retrasó la evolución de la enfermedad de forma que las células sanas no neuronales eran capaces de reducir o eliminar la toxicidad de las motoneuronas que expresaban la enzima mutada (Clement, A.M. y cols. 2003; Yamanaka, K. y cols. 2008). Estos resultados parecen dejar clara la participación de las células nerviosas no neuronales en la neurodegeneración. Sin embargo, también existen estudios que implican a las células de fuera del sistema nervioso en la etiopatogenia de la ELA, como es el caso del músculo esquelético. La importancia de la participación del músculo en la esclerosis lateral amiotrófica queda patente ya con la apreciación de alteraciones en este tejido mucho antes de la descripción de los primeros signos de degeneración de las motoneuronas. En este sentido se han observado un incremento de la captación de nutrientes, así como modificaciones en el metabolismo de carbohidratos y lípidos y alteración de las funciones mitocondriales (Dupuis, L. y cols. 2004a; Dupuis, L. y cols. 2004b; Dupuis, L. y cols. 2004c; Dupuis, L. y cols. 2009; Zhou, J. y cols. 2010). En 2006 Jokic y colaboradores realizaron un estudio en el que analizaron los niveles de Nogo-A, un inhibidor de la regeneración axonal, a nivel muscular, y observaron un incremento progresivo de su expresión desde edades presintomáticas en modelos animales SOD1-G86R (Dupuis, L. y cols. 2002; Jokic, N. y cols. 2005). Además, la eliminación genética de la expresión de Nogo-A redujo los marcadores de denervación muscular y produjo un aumento en la supervivencia de los animales. Estos autores en el mismo trabajo demostraron que el efecto de la eliminación de Nogo-A se produce a nivel de las uniones neuromusculares (Jokic, N. y cols. 2006). Por otra parte la sobreexpresión de la enzima SOD1 con distintas

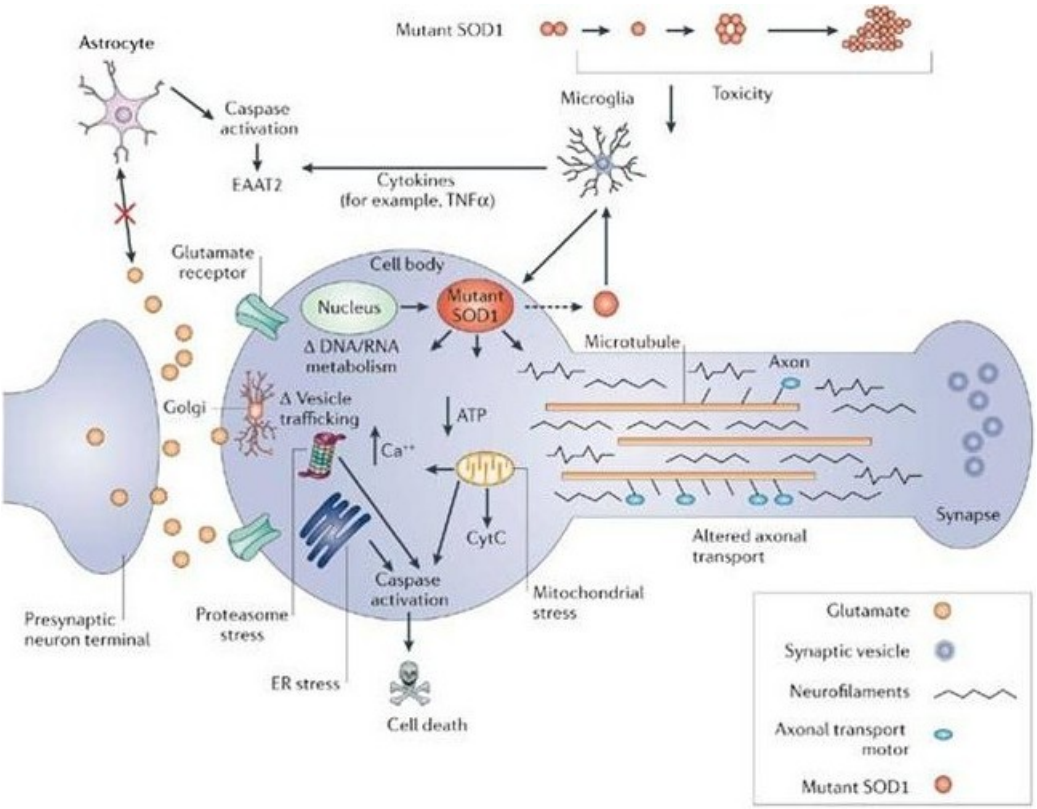


Figura 2.4: *Mecanismos propuestos para la patogenia de la ELA (Pasinelli, P. y cols. 2006).*

mutaciones exclusivamente en músculo esquelético de ratones indujo alteraciones motoras como espasticidad e hiperreflexia, pero también anomalías en las uniones neuromusculares y una axonopatía distal (Dobrowolny, G. y cols. 2008b; Wong, M. y cols. 2010). Además se ha descrito que la expresión ubicuitaria de la enzima mutada causa primero atrofia muscular, seguida de la alteración de las uniones neuromusculares y una degeneración axonal retrógrada para desembocar finalmente en la muerte de las motoneuronas (Dobrowolny, G. y cols. 2008b; Wong, M. y cols. 2010). Este patrón retrógrado y progresivo de neurodegeneración sugiere la posibilidad de que ciertas anomalías musculares precedan a la muerte de las motoneuronas. De acuerdo con estas observaciones, la aplicación de terapias dirigidas a mejorar la funcionalidad del tejido muscular, y compensar los efectos tóxicos de la SOD1 mutada sobre el mismo, ha conseguido mejorar el fenotipo de modelos animales de ELA así como prolongar su esperanza de vida. De esta forma, el ejercicio físico se ha planteado como un método paliativo ante la espasticidad y la degeneración muscular. En un principio existió cierta controversia acerca de la idoneidad de la práctica

de un ejercicio moderado en pacientes de ELAE; estas reticencias derivan del significativo porcentaje de deportistas profesionales entre los pacientes afectados por la enfermedad. Es el caso de Lou Gehrig, jugador de baseball, el boxeador Ezzard Charles o el jugador de fútbol profesional Glenn Montgomery. Sin embargo, el ejercicio físico moderado, ha estado asociado siempre al incremento de la calidad de vida y se ha demostrado que tiene efectos neuroprotectores como alivio del déficit motor, incremento de la formación neuronal y mejora de las alteraciones neurológicas en distintos procesos neurodegenerativos lo que podría estar relacionado con un incremento del factor neuroprotector Igf-1 (insulin-like growth factor 1) (van Praag, H. y cols. 1999; Mattson, M.P. 2000; Carro, E. y cols. 2001). Precisamente es el factor de crecimiento Igf-1, el que expresado exclusivamente en músculo de ratones modelo de ELA disminuyó el catabolismo de este tejido y los síntomas (Musaro, A. y cols. 2001; Kaspar, B.K. y cols. 2003; Dobrowolny, G. y cols. 2005; Dobrowolny, G. y cols. 2008a). En estos trabajos, se sugiere también que los efectos beneficiosos de esta molécula se ejercen a través del estímulo de la capacidad regenerativa y la actividad de las células madre satélite musculares (Musaro, A. y cols. 2004; Ates, K. y cols. 2007). Todo lo anteriormente expuesto hace interesante el estudio del estado y comportamiento de estas células en los modelos animales de ELA.

2.2.2. La regeneración muscular

Considerando que el trabajo que se describe en esta tesis se centra en el estudio de la regeneración muscular de las células satélite en el modelo de ELA SOD1-G93A, se ha considerado interesante realizar una revisión de los aspectos más importantes de este proceso. La regeneración muscular comprende dos fases; una primera fase degenerativa que se caracteriza por una necrosis de las fibras musculares dañadas y una activación de células mononucleares. La ruptura del sarcolema por el daño incrementa la permeabilidad en la fibra muscular, lo que permite la salida de proteínas como la creatin kinasa citosólica al suero. Este aumento de la permeabilidad, propicia además un incremento de la captación de calcio que provoca una pérdida de la homeostasis en dicho elemento, y por último una proteólisis por activación de proteasas como las calpainas, dependientes de calcio; y que degradan las proteínas de las miofibras y el citoesqueleto. El proceso de activación de células mononucleares comprende principalmente células inflamatorias y miogénicas que se activan gracias a señales quimiotácticas procedentes del músculo dañado.

En segundo lugar se produce la fase regenerativa, cuyo principal proceso es la

proliferación celular. Las células miogénicas activadas en la fase anterior proliferan, se diferencian y por último se fusionan a miofibras ya existentes (si es suficiente una reparación), o unas con otras para generar nuevas miofibras (si es necesaria una regeneración). En este proceso destaca la proteína m-calpaina, que participa en la reorganización del citoesqueleto durante la fusión de las células progenitoras miogénicas en diferenciación. Histológicamente, las miofibras recientemente formadas se caracterizan en su sección transversal por presentar un pequeño tamaño nuclear y en posición central, ser de carácter basofílico por una intensa síntesis protéica y una expresión de la cadena pesada de la miosina (MHC). En su sección longitudinal, las miofibras aisladas presentan núcleos centrales y destaca la presencia de miofibras ramificadas, en proceso de fusión. En las miofibras ya maduras, sin embargo, se observa un mayor diámetro y núcleos desplazado a la periferia y son indistinguibles de las que no sufrieron daño (Charge, S.B. y cols. 2004). Las células satélite musculares son unas células mononucleadas localizadas en el espacio entre la lámina basal y el plasmalema de la miofibra (Mauro, A. 1961). Poseen un núcleo de tamaño reducido y un mayor contenido en heterocromatina respecto a los núcleos de las miofibras, así como un bajo contenido en orgánulos; todas ellas características de quiescencia (Morgan, J.E. y cols. 2003). Las células satélite provienen de progenitores localizados en el dermomiotomo de los somitos caracterizados por ser células PAX3 y PAX7 positivas (Kassar-Duchossoy, L. y cols. 2005; Relaix, F. y cols. 2005), y se identifican mediante en el empleo de anticuerpos para marcadores específicos por inmunohistoquímica, así como microscopía electrónica (Zammit, P.S. y cols. 2004; Relaix, F. y cols. 2006). El daño muscular generado por traumatismos, enfermedades, denervación o por el ejercicio físico intenso, produce una activación de las células satélite musculares. Esta activación se localiza no solamente en la zona dañada sino en toda la miofibra, y se extenderá a las fibras musculares y músculos adyacentes si el daño ha afectado al tejido conectivo. Tras la activación, se producen múltiples ciclos de proliferación, momento en el cual estas células pasan a denominarse células precursoras miogénicas o mioblastos. En este momento se activan en la miofibra diversas cascadas moleculares que incrementan la expresión de los llamados factores de regulación miogénicos (MRFs), concretamente de *Myod1* y *Myf-5*. Tras esta proliferación, los mioblastos pasan bien a diferenciarse, momento en el cual dejan de expresar *Pax7* y *Myf-5* y comienzan a expresar miogenina y más tardíamente *Mrf4* para posteriormente fusionarse entre ellos o con las miofibras no dañadas, para regenerar el músculo, o bien regresan a formar parte del pool de célu-

las satélite quiescentes si dejan de expresar *Myod1* y continúan expresando *Myf-5* (Charge, S.B. y cols. 2004; Collins, C.A. y cols. 2005)(Figura 1.5).

Los MRFs son factores de transcripción que regulan genes relacionados con el músculo (creatin quinasa, cadena ligera de la miosina, receptor de acetil colina...), además como hemos comentado anteriormente, activan la transcripción y diferenciación de mioblastos en miotubos funcionales. Se trata de un tipo de proteínas que en su estructura cuentan con un dominio bHLH (basic hélix-loop-helix), es decir dos α-hélices unidas por un lazo.

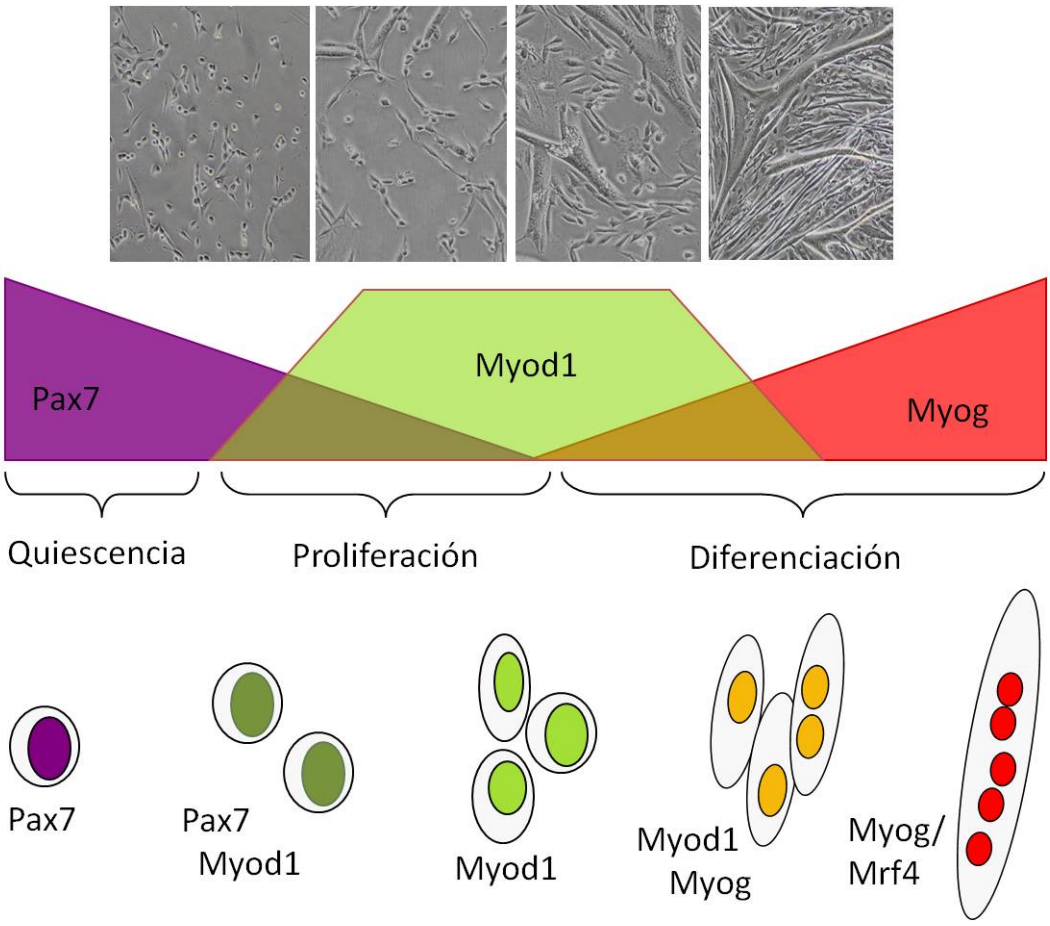


Figura 2.5: *Representación esquemática de la miogénesis en adultos (modificado de (Le Grand, F. y cols. 2007).*

Estas proteínas para su actividad forman heterodímeros con otras proteínas del grupo bHLH que pertenecen a la clase I o E (Massari, M.E. y cols. 2000). La capacidad de unión al DNA radica en la flexibilidad del lazo para plegarse, de forma que

las dos hélices de cada monómero forman una horquilla que interacciona con adenina y citosina de secuencias consenso CANNTG. Estas secuencias están localizadas en E-box de las regiones promotoras y enhancer de los genes sobre los que actúan estos factores (Wyzykowski, J.C. y cols. 2002). En condiciones ambientales restrictivas, este proceso se ve inhibido a través de modificaciones postranscripcionales de los MRFs, que generan una disminución de la formación de heterodímeros, o una menor capacidad de unión al DNA, un aumento del turnover de las proteínas MRFs, o una inducción de proteínas de la familia Id (Inhibidores de la diferenciación) (Wyzykowski, J.C. y cols. 2002). Todos los estudios presentados nos llevan a pensar que el músculo esquelético es una diana directa del daño inducido por la SOD1 en pacientes y modelos animales de ELA. Y que éste tejido estaría afectado en fases previas al daño y pérdida crítica de motoneuronas. Ahora bien, si este tejido desempeña un papel importante en la etiopatogenia de la enfermedad podrían plantearse terapias eficaces dirigidas al mismo, y sustancias que estimulen la regeneración miogénica y en concreto la actividad de las células satélite. Por todo ello, consideramos que estudios que permitan conocer el estado y comportamiento de dichas células a lo largo de la enfermedad son de gran interés antes de realizar ensayos terapéuticos. En este sentido trabajos pioneros en este campo describieron el incremento de la expresión de genes relacionados con el control del ciclo celular, el crecimiento y la diferenciación y miogénesis en el músculo de animales modelo de ELA en fases previas a la parálisis (Gonzalez de Aguilar, J.L. y cols. 2008). Muy recientemente ha surgido otro estudio, en este caso en pacientes, que analiza la capacidad miogénica de las células satélite de enfermos de ELA esporádica *in vitro*. En este trabajo se observa una menor capacidad proliferativa y un retraso en la diferenciación de las células satélite provenientes de enfermos en base a una mayor expresión del isotipo embrionario de la cadena pesada de miosina (MHC). Por otra parte, se observa una morfología anormal de dichas células que se corrobora con ensayos de Western blot mediante cuantificación de p16, un marcador de senescencia y estrés celular (Pradat, P.F. y cols. 2011). La dificultad para la obtención de suficientes muestras de pacientes y en distintos momentos de la enfermedad, y la variabilidad en la presentación de los síntomas impidió, en este trabajo, el estudio de grupos homogéneos en edad, sexo, presentación clínica o fase de la enfermedad, así como la comparación de las células satélite obtenidas de varios músculos. Sin embargo, la influencia de estos factores en el comportamiento de estas células *in vitro*, como se ha demostrado en el manuscrito 3 de esta tesis, hace necesario con-

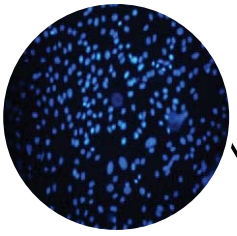
siderarlos. Por lo tanto, aunque importantes, los resultados que hasta ahora se han descrito en pacientes necesitan ser complementados con estudios en modelos animales que permitan crear grupos homogéneos de donantes de las células en edad, sexo y presentación clínica, así como evaluar distintos tipos de músculos.

2.2.3. Modelos experimentales de ELA

Muchos han sido los esfuerzos realizados para encontrar un modelo que reproduzca las manifestaciones clínicas y etiopatogénicas observadas en ELA incluyendo tanto ensayos *in vitro,* como modelos animales *in vivo.* En estudios *in vitro* se han utilizado cultivos primarios de astrocitos en solitario o en co-cultivo con motoneuronas que expresaban SOD1 humana mutada (Rao, S.D. y cols. 2003; Tortarolo, M. y cols. 2004; Nagai, M. y cols. 2007). Además se han desarrollado cultivos organotípicos derivados de la médula espinal, con superóxido dismutasa 1 inhibida o con distintas mutaciones (Rothstein, J.D. y cols. 1994; Durham, H.D. y cols. 1997). Finalmente, la línea celular NSC-34, se transfectó con vectores que contienen el gen de la superóxido dismutasa 1 mutada y es considerada como un modelo celular de ELA (Gabbianelli, R. y cols. 1999; Atkin, J.D. y cols. 2006; Raimondi, A. y cols. 2006; Gomes, C. y cols. 2008). A pesar de la utilidad de estos modelos *in vitro*, la compleja etiopatogenia de la ELA, con distintos sistemas involucrados en su desencadenamiento y progresión, hace indispensable el uso modelos *in vivo* en los que esté presente el sistema inmunitario y hormonal. Los primeros modelos *in vivo* se centraban en el estudio de animales con degeneraciones de la motoneurona por mutaciones espontáneas. El más conocido es el ratón denominado *wobbler* que porta una mutación sin sentido del locus *wr* que codifica la proteína vacuolar vesicular sorting 54 (Vsp54) (Schmitt-John, T. y cols. 2005; Perez-Victoria, F.J. y cols. 2010). Además, existen otros ratones como el *pmn* (Schmalbruch, H. y cols. 1991; Martin, N. y cols. 2002; Fischer, L.R. y cols. 2004; Schaefer, M.K. y cols. 2007), *wst/wst (wasted)* (Kaiserlian, D. y cols. 1985; Lutsep, H.L. y cols. 1989), *nmd* (Cook, S.A. y cols. 1995; Cox, G.A. y cols. 1998; Maddatu, T.P. y cols. 2004), *mnd* (Messer, A. y cols. 1986; Messer, A. y cols. 1992) y *mdf* (muscle deficient)(Blot, S. y cols. 1995; Kaestner, K.H. y cols. 1995; Schmidt, W.M. y cols. 2007). Por otra parte se han utilizado distintos mecanismos para inducir daño a las motoneuronas *in vivo* como inoculación de agonistas de alguno de los tipos de receptores del glutamato (Corona, J.C. y cols. 2004) o de sustancias químicas neurotóxicas (Wakayama, I. y cols. 1996; Segura Aguilar, J. y cols. 2004; Vasudevaraju, P. y cols. 2008). También se indujo

neurodegeneración mediante fenómenos de autoinmunidad al inocular cobayas con motoneuronas purificadas o con el homogenado procedente del asta ventral de la médula espinal bovina, de esta forma se generaron la enfermedad de la motoneurona alérgica experimental (EAMND) (Engelhardt, J.I. y cols. 1989) y la enfermedad experimental autoinmune de la materia gris (EAGMD) respectivamente (Engelhardt, J.I. y cols. 1990; Tajti, J. y cols. 1991). Gracias al descubrimiento de las mutaciones en la enzima SOD1 asociadas al padecimiento de la enfermedad, se generaron los animales modificados genéticamente, que en la actualidad se consideran como los mejores modelos de la ELA humana *in vivo*. Estos animales se han generado por mutación, sobre-expresión o interrupción de los genes más comúnmente alterados en la ELA familiar humana. Es el caso de los ratones que portan alteraciones en genes relacionados con el transporte axonal como la periferina (Beaulieu, J.M. y cols. 1999a; Beaulieu, J.M. y cols. 1999b), dinactina (LaMonte, B.H. y cols. 2002; Hafezparast, M. y cols. 2003), kinesina (*Kif1b*) (Zhao, C. y cols. 2001), dineína o las cadenas de los neurofilamentos (*NF-H*) (Cote, F. y cols. 1993; Xu, Z. y cols. 1993). También en genes relacionados con factores de crecimiento como gen de factor de crecimiento vascular endotelial (*Vegf*) (Matsuzaki, H. y cols. 2001; Oosthuyse, B. y cols. 2001; Tuder, R.M. y cols. 2003; Greenberg, D.A. y cols. 2004), factor neurotrófico derivado de cerebro (*Bdnf*) (Jones, J.M. y cols. 1993; Klein, R. y cols. 1993; Klein, R. 1994), el factor neurotrófico ciliar (*Cntf*) (Masu, Y. y cols. 1993; Orrell, R.W. y cols. 1995) o la Alsina (*Als2*) (Hadano, S. y cols. 2001; Yamanaka, K. y cols. 2003; Cai, H. y cols. 2005). De entre todos, el modelo más utilizado *in vivo* es el ratón que sobre-expresa la enzima superóxido dismutasa 1 humana mutada. Se han generado diversas cepas de ratones que portan SOD1 humana con distintas mutaciones y número de copias del transgén, entre las más utilizadas se encuentran la G93A (Gurney, M.E. y cols. 1994), G85R (Bruijn, L.I. y cols. 1997), G37R (Wong, P.C. y cols. 1995) y H46R/H48Q (Wang, J. y cols. 2002). Las distintas mutaciones originan variaciones en la estabilidad de la enzima y en su actividad dismutasa, desde la inhibición total de ésta hasta la no modificación (Williamson, T.L. y cols. 1999; Jonsson, P.A. y cols. 2004). En la actualidad una de las cepas más utilizadas como modelo de ELA es la que porta la mutación G93A, en concreto la línea de alto número de copias (G1H) que ha sido bien caracterizada (Gurney, M.E. y cols. 1994; Miana-Mena, F.J. y cols. 2005). Por esto, fue el utilizado en los experimentos para la realización de esta Tesis Doctoral y nos centraremos en su descripción de forma esquemática. En el ratón SOD1-G93A, se ha descrito anomalías

como la presencia de agregados de SOD1 mutada, la vacuolización de las motoneuronas o la fragmentación del aparato de Golgi ya a los 30 días de edad (Mourelatos, Z. y cols. 1996; Johnston, J.A. y cols. 2000; Turner, B.J. y cols. 2003) (Chiu, A.Y. y cols. 1995); y la primera detección de pérdida de unidades motoras a los 47-50 días (Kennel, P.F. y cols. 1996; Frey, D. y cols. 2000; Hegedus, J. y cols. 2007). Los primeros déficits motores claros aparecen a las 8 semanas (Wooley, C.M. y cols. 2005) aunque el inicio sintomático se establece alrededor de los 90-100 días de edad como un ligero temblor acompañado de debilidad muscular en una o más extremidades y que puede ir acompañado de paresis incompleta. La progresión se produce como parálisis ascendente de las extremidades anteriores hasta que en las fases terminales, alrededor de 120 días de edad, se genera un estado de cuadriplegia severa (Gurney, M.E. y cols. 1994; Chiu, A.Y. y cols. 1995). A nivel citopatológico, las manifestaciones más características son tres; formación de vacuolas intracitoplasmáticas neuronales, inclusiones neuronales positivas a SOD1 (Stieber, A. y cols. 2000; Kato, S. 2008) y pérdida selectiva de motoneuronas en la médula espinal y tronco del encéfalo. Respecto al comienzo de este último, existe gran controversia, algunos autores sitúan las primeras evidencias en los 100 días (Kato, S. 2008), mientras otros detectan la pérdida de uniones neuromusculares y axones en el asta ventral de la médula a partir de 47 días, y de cuerpos neuronales a partir de 80 días (Kennel, P.F. y cols. 1996; Fischer, L.R. y cols. 2004). En este sentido, se ha demostrado que el tipo de fibra muscular que inervan es condicionante clave para su susceptibilidad a la acción de la SOD1 mutada. De esta forma, la denervación y pérdida de unidades motoras se produce de forma más temprana, en torno a 40 días en las motoneuronas que inervan fibras musculares de contracción rápida (tipo IIB). Por el contrario, las que inervan fibras de tipo lento (I y IIa) mantienen estos parámetros inalterados hasta los 90 días de edad, momento en el que se establece el comienzo de la fase sintomática de la enfermedad (Hegedus, J. y cols. 2007; Hegedus, J. y cols. 2008). En esta tesis nos ocupamos de este aspecto y caracterizamos el estado y comportamiento de las células satélite musculares derivadas del ratón modelo de ELA SOD1-G93A tanto *in vivo* como *in vitro* desde las fases postnatales más tempranas hasta el estadio terminal de la enfermedad. Asimismo incluimos en nuestro estudio parámetros como la edad del animal, el sexo o el tipo de músculo que se han descrito como factores que influencian el desarrollo de la ELA.



OBJETIVOS

2.2.4. Objetivos

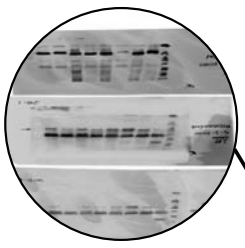
El objetivo general planteado en la presente memoria de Tesis Doctoral es conocer el estado de las células satélite musculares y de sus factores de regulación miogénica en el modelo murino SOD1-G93A de ELA a lo largo de la enfermedad. Para alcanzar este objetivo general se han planteado los siguientes objetivos específicos:

1. Determinar los niveles de transcritos y proteína del marcador de células satélite *Pax7* y los factores de regulación miogénica (MRFs) en animales modelo de ELA SOD1-G93A a lo largo de la enfermedad respecto a sus controles.

2. Cuantificar el número de células madre satélite musculares (SMSCs), su capacidad de activación y su evolución a lo largo de la enfermedad en las distintas fibras (de contracción rápida y lenta) *in vivo*.

3. Evaluar si las alteraciones en la cantidad y estado de activación de las células satélite musculares en las miofibras de los ratones SOD1-G93A a lo largo de la enfermedad son debidas a características intrínsecas de las mismas o a factores externos, mediante técnicas de cultivo celular.

4. Conocer si existe alteración en la capacidad de proliferación de las células satélite de animales SOD1-G93A en la fase neonatal cuando no existen evidencias de denervación y el periodo de acción de la enzima mutada es reducido.



RESUMEN DE LOS MANUSCRITOS

2.2.5. Manuscritos

2.2.5.1. Manuscrito 1 Alteración en la expresión de los factores de regulación miogénica en el músculo esquelético de un modelo murino de esclerosis lateral amiotrófica

El objetivo de este trabajo fue cuantificar los niveles de transcritos y proteína del marcador de células satélite *Pax7* y los factores de regulación miogénica (MRFs) *Myod1*, *Myf5* y miogenina en animales modelo de ELA SOD1-G93A en las distintas fases de la enfermedad, 40 días (estado presintomático temprano), 60 días (estado presintomático tardío), 90 días (comienzo de los síntomas) y 120 días (fase terminal) y compararlos con los de sus controles no transgénicos. Por otra parte, para determinar si las modificaciones de estos factores eran paralelas al daño muscular oxidativo se cuantificó la expresión de *Rrad* (Ras-related associated with diabetes). Asimismo, para conocer el efecto de la denervación en estos factores se realizó una axotomía del nervio ciático a un grupo de ratones no transgénicos de 60 días y se cuantificaron los niveles de estos factores en el músculo esquelético 2 semanas tras la operación. Además, para monitorizar el inicio y progresión de la denervación en la enfermedad se cuantificó la expresión del receptor de la acetilcolina alfa 1(*Chrna1*) en animales SOD1-G93A comparándolo con sus controles no transgénicos y con los animales denervados. Para ello se extrajo el tejido de los principales grupos musculares de las extremidades posteriores (cuádriceps, gastrocnemio y sóleo) de ratones macho SOD1-G93A, denervados y sanos. Posteriormente se procedió al procesado de las muestras para la obtención de RNAm y proteína de las mismas y se cuantificó la expresión de los factores mencionados mediante técnicas de reacción en cadena de la polimerasa (PCR) en tiempo real y Western blot respectivamente. Los resultados mostraron en primer lugar un incremento progresivo de *Rrad* y *Chrna1* en los animales modelo comenzando el primero en fases presintomáticas (60 días de edad) y el segundo con el inicio de los síntomas (a los 90 días de edad), lo que podría indicar la existencia de un daño muscular previo a la denervación y pérdida de motoneuronas. En segundo lugar se apreció la inducción de *Pax7* y los factores de regulación miogénica especialmente a partir de la aparición de los síntomas (90 días de edad) y en fases terminales (120 días de edad). Estos resultados concuerdan con los observados en los animales denervados y el incremento de *Chrna1* lo que sugiere que la respuesta regenerativa del músculo de los animales SOD1-G93A a nivel de transcripción viene determinada por la denervación y pérdida de conexiones neuromusculares. Por otra parte la cuantificación de PAX7 y los MRFs a

nivel proteínico reveló una respuesta truncada de estos factores a nivel traduccional. Es especialmente significativo el caso de las fases terminales de la enfermedad (120 días de edad) donde a pesar de observarse un incremento máximo de trascritos de *Pax7*, *Myod1*, *Myf5* y miogenina no se corresponden con una mayor cantidad de proteína. En este trabajo se discuten las posibles causas de esta discordancia. Se sugiere que la característica regulación de los MRFs mediante fosforilaciones, su hetero-oligomerización con proteínas E y la existencia de factores inhibidores de esta oligomerización puede favorecer este desacoplamiento entre RNAm y proteína. Por otra parte, el incremento de la actividad del proteasoma o la reducción de la estabilización postranscripcional de estos factores a causa del daño oxidativo y el estrés del retículo endoplásmico contribuirían también a la reducción de los niveles proteínicos de PAX7 y los MRFs. En conclusión, en este trabajo se aprecia una respuesta al daño oxidativo previa al incremento de los marcadores de denervación en los ratones modelo SOD1-G93A. Además, se observa la inducción de una respuesta regenerativa a nivel de transcritos en paralelo al daño muscular y a la denervación existiendo, sin embargo una discordancia entre el RNAm y los niveles proteínicos. La apreciación de una respuesta regenerativa en el músculo de los animales modelo de ELA SOD1-G93A y de un daño muscular previo a la detección de la pérdida de conexiones neuromusculares, nos llevó a plantearnos el estudio pormenorizado del estado del pool de células madre satélite musculares responsables de la reparación y regeneración muscular. Con este propósito planteamos el estudio que recoge el manuscrito 2.

2.2.5.2. Manuscrito 2 Cantidad y activación de las células satélite musculares asociadas a miofibras en un modelo murino de esclerosis lateral amiotrófica

Nuestro objetivo en este trabajo fue cuantificar el número de células madre satélite musculares (SMSCs), su capacidad de activación y su evolución a lo largo de la enfermedad en el modelo animal de esclerosis lateral amiotrófica SOD1-G93A respecto a sus controles no transgénicos. En ELA existe una mayor vulnerabilidad de las unidades motoras que inervan fibras musculares de contracción rápida ("fast") y metabolismo glucolítico, por este motivo, el estudio se realizó en dos músculos diferentes; extensor digital largo (EDL), compuesto casi en su totalidad por fibras "fast" (IIb y IIa) y sóleo (SOL), con un 60% de fibras de contracción lenta ("slow") oxidativas (I) y 40% tipo IIa. Al igual que en el trabajo anterior se establecieron cuatro puntos de análisis en la evolución de la enfermedad en este modelo, 40, 60, 90 y 120 días de edad (ver manuscrito 1). Para la determinación del número de células satélite de ambos músculos se realizó el aislamiento de sus miofibras individualizadas y el marcaje inmunohistoquímico para la proteína PAX7, específica de este tipo celular. Posteriormente se procedió del mismo modo para la proteína MYOD1 expresada en las células satélite musculares activadas como se ha explicado con anterioridad. Finalmente se realizó un contaje mediante microscopía de fluorescencia del número de núcleos marcados para cada proteína en cada miofibra y se compararon los resultados de las miofibras de cada músculo y edad en los animales SOD1-G93A y sus correspondientes controles no transgénicos. Los resultados mostraron ya en fases presintomáticas tempranas (40 días) una disminución en el número de células satélite (PAX7 positivas) en las miofibras de los animales modelo en comparación con los sanos. Este resultado coincidió en las miofibras de ambos músculos. Posteriormente en la fase presintomática tardía (60 días) se observó que las miofibras del músculo EDL de ratones enfermos habían incrementado ligeramente su número de células PAX7 positivas igualando los niveles de las miofibras de animales sanos. Sin embargo, las de sóleo mostraban un número mucho mayor de estas células en relación con sus controles no transgénicos. A diferencia de la fase anterior el músculo EDL de los animales SOD1-G93A a 90 días de edad presentó un incremento en el número de SMSCs, mientras que las miofibras del sóleo presentaron una disminución dramática en su contenido en estas células. Finalmente en la fase terminal de la enfermedad las fibras del músculo sóleo presentaron un incremento del pool de SMSCs mientras que las de EDL permanecieron similares a los

animales sanos. En cuanto al estado de activación de estas células (células MYOD1 positivas), el primer signo se observó a los 60 días con un incremento del número de células MYOD1 positivas por miofibra en el músculo sóleo de animales SOD1-G93A en paralelo al incremento de células PAX7 positivas. En la fase sintomática las miofibras de ambos músculos presentaron mayor número de células activadas, para volver a disminuir en las fases terminales de la enfermedad. En resumen, estos datos concuerdan con una alteración de las células satélite musculares previa a la pérdida de unidades motoras y fuerza de contracción. Por lo tanto es previsible que la disminución temprana del pool de células satélite refleje un efecto directo de la toxicidad de la enzima mutada sobre el tejido muscular. Por otra parte hemos observado una evolución distinta en el pool de células satélite y su capacidad de activación entre las miofibras de animales SOD1-G93A derivadas de EDL y sóleo; siendo estas últimas las que reaccionan antes incrementando su número y su activación y también las fibras más resistentes a la enfermedad. Este trabajo demuestra el compromiso de las células satélite musculares en la enfermedad desde las fases más tempranas y pone de manifiesto que la mayor vulnerabilidad de las unidades motoras "fast" podría deberse a una respuesta más tardía de sus células satélite. Sin embargo queda por discernir 1) si la alteración temprana de estas células está causada por señales procedentes del nicho celular o es consecuencia directa de la acción de la SOD1 sobre las mismas y 2) si la heterogeneidad dependiente del tipo de fibra viene determinada por señales procedentes de las distintas motoneuronas que los inervan, por el tejido muscular o refleja una diferencia intrínseca en la respuesta de estas células a la toxicidad de la SOD1 mutada. Para resolver esta cuestión nos planteamos la posibilidad de extraer las células musculares de los animales modelo y ponerlas en cultivo, aislándolas, de este modo de las posibles señales exógenas que puedan influir en su comportamiento. Este trabajo ha dado lugar a los manuscritos 5 y 6 que describiremos más adelante. Sin embargo, antes de abordar el diseño experimental de estos trabajos nos planteamos dos cuestiones, la primera, la posible influencia de otros parámetros fisiológicos como el sexo, la edad del animal o el tipo de músculo sobre el comportamiento de sus células satélite en animales sanos *in vitro*, esto nos sirvió para determinar si es necesario incluir controles específicos para cada edad y sexo. Para responder a esta pregunta analizamos la capacidad de proliferación, diferenciación y expresión de factores de regulación miogénica de células satélite musculares obtenidas de ratones sanos macho y hembra, desde los primeros días de vida hasta la fase adulta y tomando los músculos extensor digital

largo y sóleo. Este trabajo dio lugar al manuscrito 3. La segunda cuestión que se nos planteó deriva de que la mayoría de los análisis moleculares que se presentan en los trabajos con las células en cultivo *in vitro* se centran en estudios de expresión génica mediante PCR en tiempo real. Para la correcta interpretación de los datos proporcionados por esta técnica se hace necesario referir los resultados del gen problema a los de otro gen normalizador o "housekeeping" que por definición ha de estar expresado constitutivamente en todas las muestras a analizar. Ante la falta de trabajos que probasen esta característica en las células satélite de los animales modelo, procedimos al análisis de la estabilidad de 6 genes candidatos en cultivos de estas células procedentes de animales sanos y modelo SOD1-G93A de ambos sexos y músculos de interés y desde el nacimiento hasta la edad adulta. Finalmente incluimos en el estudio muestras de animales axotomizados ya que este fenómeno es también propio de la enfermedad y nos servirán de control en estudios posteriores para discernir los efectos propios de la enzima mutada y los derivados de la pérdida de inervación. Este trabajo dio lugar al manuscrito 4.

2.2.5.3. Manuscrito 3 Proliferación de las células madre satélite musculares en función del sexo, la edad y el tipo de fibra del que proceden

El objetivo de este trabajo fue determinar la influencia del sexo, la edad y el tipo de fibra muscular del donante en la capacidad de proliferación y expresión de *Pax7* y los factores de regulación miogénica (MRFs) *Myod1* y miogenina *in vitro* de la células madre satélite musculares. Para cumplir con este objetivo, obtuvimos las células madre de los músculos extensor digital largo (fibras de contracción rápida) y sóleo (fibras de contracción lenta) de ratones macho y hembra de 7 (neonatos), 40 (joven), 60 (jóvenes adultos) y 120 (adultos) días de edad. Inmediatamente tras su extracción se pusieron en cultivo en condiciones que favorecían su proliferación y diferenciación. Desde los 3.5 hasta los 7.5 días tras la siembra diariamente se realizó un recuento del número de células presentes en el cultivo de cada grupo de edad, sexo y origen. Por otra parte, para el análisis molecular, a días 5.5 (representativo de la capacidad de proliferación) y 7.5 de cultivo (formación patente de miotubos), se extrajo el RNAm de las células de cada grupo y se analizaron mediante PCR en tiempo real a día 5.5 de cultivo *Pax7*, un marcador de células satélite y *Myod1*, un indicador de activación de dichas células. Finalmente, a día 7.5 se cuantificó la expresión de *Myod1*, miogenina y *Mrf4*, los dos últimos marcadores de diferenciación de las mismas.

Los resultados mostraron que la proliferación *in vitro* de las células satélite derivadas del músculo extensor digital largo de ratones macho y hembra era más rápida que la de las células obtenidas de sóleo desde las fases neonatales (7 días de edad) hasta los jóvenes adultos (60 días de edad). Por otra parte las células obtenidas de machos también presentaban una mayor proliferación en comparación con las hembras en ambos tipos de fibras y durante todo el desarrollo excepto en neonatos. En consistencia con estos resultados los cultivos obtenidos de los machos presentaron en jóvenes y jóvenes adultos una disminución de *Pax7* y un incremento de *Myod1*, miogenina y *Mrf4* lo que indicaría un estímulo mayor del programa miogénico en este grupo también a nivel molecular. En este manuscrito discutimos además las posibles causas de esta heterogeneidad en la proliferación de las células satélite entre ambos sexos y tipos de fibras musculares. Especulamos con una posible estimulación de las células de los machos a partir de la esteroidogénesis testicular, sin embargo que la influencia hormonal persista aún después de haber extraído las células de su nicho parece poco factible aunque no imposible. Por otra parte sugerimos una diferencia innata en la capacidad de regeneración de las células en ambos sexos que

otros autores han comprobado *in vivo*. Finalmente las diferencias pueden estas asociadas a una diferente proporción de células satélite HPC (de alta proliferación) y LPC (de baja proliferación) en ambos sexos. Por otro lado, la alta demanda de fibras de contracción rápida capaces de generar masa muscular durante el crecimiento justificaría su mayor proliferación. En adulto, sin embargo, las fibras de contracción lenta, por otra parte las más frecuentemente reclutadas en la actividad diaria, presentarían mayor proliferación, lo que además concuerda con el mayor número de células satélite asociadas a miofibras de contracción lenta descrito en el manuscrito 2 y en la bibliografía consultada. Sea cual sea la causa de la heterogeneidad de género, edad y tipo de fibra en la capacidad proliferativa de las células satélite *in vitro*, se demostró que era necesario incluir controles no transgénicos de cada uno de estos grupos para compararlos con los animales modelo SOD1-G93A. Por otra parte estos factores pueden condicionar el éxito de terapias celulares y por lo tanto deben tenerse en el diseño experimental de los ensayos.

2.2.5.4. Manuscrito 4 Expresión de genes housekeeping en cultivos de células miogénicas en modelos animales de neurodegeneración y denervación

Nuestro objetivo en este trabajo fue la validación de seis genes candidatos a housekeeping en cultivos de células satélite derivadas de ratones de ambos sexos, de músculos compuestos por fibras de tipo "fast" glicolíticas y "slow" oxidativas (ver manuscrito 2), desde los primeros días tras el nacimiento hasta la edad adulta así como comprobar la estabilidad de estos genes en cultivos miogénicos de animales sanos y modelos de neurodegeneración (SOD1-G93A) y denervación. Para esto se aislaron las células satélite de los grupos de ratones anteriormente mencionados y se pusieron en cultivo *in vitro* durante 7.5 días para permitir su proliferación y diferenciación. Posteriormente se extrajo el RNAm de estos cultivos y se procesó para el análisis de la expresión de *Rn18s* (RNA ribosomal 18S), *Actb* (beta-actina), *Gapdh* (gliceraldehido-3-fosfato deshidrogenasa), *Sdha* (subunidad A del complejo succinato deshidrogenasa), *B2m* (beta-2 microglobulina) y *Hprt1* (hipoxantina guanina fosforibosil transferasa) mediante PCR en tiempo real. Para el análisis de la estabilidad de la expresión de estos genes se utilizó en software geNorm, que proporciona un índice M que representa la variación de cada gen respecto de la del resto de los genes analizados. Además calcula el número mínimo de genes necesarios para crear un factor de normalización fiable. De esta forma se observó que el factor que mayor variabilidad introducía en estos genes es la edad del animal donante de las células, de modo que en los análisis que comprendían muestras de distintas edades se obtenían valores de M elevados, incluso el gen *Rn18s* superaba el valor de 1.5 establecido como umbral máximo aceptable para un "housekeeping". Por otra parte, en los análisis llevados a cabo con muestras de la misma edad, la variabilidad de los genes era reducida independientemente del sexo, tipo de músculo o fenotipo de los donantes, lo que refleja la baja influencia de estos factores en la estabilidad de los genes estudiados. Al realizar el cálculo del número de genes necesarios para crear un factor de normalización adecuado se observó que en las muestras de cultivos miogénicos de donantes del mismo sexo, edad y tipo muscular, era recomendable un mínimo de dos genes. Sorprendentemente el fenotipo neurodegenerativo o la denervación del músculo no inducía variación en la expresión de estos genes presentando valores de M muy bajos. En resumen, en este trabajo demostramos que el factor que mayor influencia tiene en la expresión de los genes estudiados en los cultivos de células satélite musculares es la edad del donante. Además validamos

la estabilidad de todos ellos en análisis cuyas muestras procedan de animales de la misma edad. Finalmente recomendamos el uso de un mínimo de 2 de estos genes para el cálculo del factor de normalización. Siguiendo estas premisas en los análisis de expresión génica de los manuscritos 5 y 6 se utilizó la media geométrica de tres housekeeeping para asegurar un buen factor de normalización ya que se realizaron estudios comparando las muestras de animales SOD1-G93A o denervados con sus correspondientes controles del mismo sexo, edad y tipo de músculo.

2.2.5.5. Manuscrito 5 Alteración de la capacidad de proliferación *in vitro* de las células satélite musculares en el modelo animal SOD1-G93A

El presente trabajo se planteó con el objetivo de determinar si las alteraciones en la cantidad y estado de activación de las células satélite musculares en las miofibras de los ratones SOD1-G93A modelos de ELA a lo largo de la enfermedad (manuscrito 2) viene predeterminada por señales procedentes del nicho celular o es consecuencia directa de la acción de la SOD1 mutada sobre las mismas. Además se propuso conocer si las diferencias entre las células de músculos compuestos por fibras de tipo "fast" glucolíticas y "slow" oxidativas y en la mayor vulnerabilidad de las primeras a la enfermedad está causada por diferencias intrínsecas en las células y en su capacidad de respuesta al daño de la SOD1 o es consecuencia de las distintas motoneuronas que inervan a ambos tipos de fibras. Para ello, se extrajeron las células satélite de los músculos extensor digital largo y sóleo de animales macho modelo SOD1-G93A y controles no transgénicos en las distintas fases de la enfermedad (ver manuscrito 2). Dichas células se pusieron en cultivo *in vitr*o en condiciones que favorecían su proliferación y diferenciación. Diariamente desde los 3.5 hasta los 7.5 días se realizó un recuento del número de células de cada grupo y se compararon los datos de los ratones modelo con los de no transgénicos de cada músculo. Además en los días 5.5 (cuando la proliferación de las células era patente) y 7.5 (cuando se empezó a observar la formación de pequeños miotubos), se lisaron las células y se extrajo el RNAm. Posteriormente las muestras se procesaron y analizaron mediante PCR en tiempo real para determinar la expresión génica de *Pax7* (marcador de células satélite) y *Myod1* (marcador de activación de las células) (a los 5.5 días) y de miogenina (marcador de diferenciación, a los 7.5 días de cultivo). En general los resultados mostraron un menor ratio de proliferación en células satélite musculares de los animales SOD1-G93A en comparación con los animales no transgénicos. Esta tendencia se mantiene desde la fase presintomática temprana hasta la terminal y en ambos músculos estudiados. Sin embargo, en la fase sintomática, coincidiendo con el comienzo de la fase crítica de denervación, la proliferación de estas células se ve incrementada en comparación con las de los animales control. Este resultado es similar al observado en cultivos de células satélite de animales sanos denervados. A nivel molecular los resultados corroboraron mayoritariamente lo observado en proliferación y en las fases presintomáticas los tres factores analizados se encontraron disminuidos en los cultivos de animales SOD1-G93A de los dos músculos estudiados. Posteriormente, en la fase sintomática se observó un incremento de los marcadores

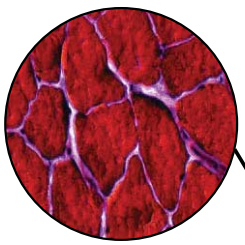
de activación y diferenciación hasta igualar o superar los de los cultivos de animales control. Finalmente en la fase terminal, *Myod1* volvió a disminuir en las células derivadas de sóleo y miogenina en ambos músculos de los animales SOD1-G93A, lo que corroboró el descenso observado también en la proliferación en esta fase. En resumen, los resultados obtenidos en este trabajo nos llevan a pensar que la capacidad proliferativa de las células de los animales SOD1-G93A está disminuida desde las fases presintomáticas de la enfermedad. Estas observaciones apoyan la teoría de que la patología muscular en los ratones modelo de ELA SOD1-G93A no deriva exclusivamente del fallo de las motoneuronas que los inervan sino de la toxicidad de la enzima mutada sobre este tejido y en este caso sobre sus células madre. Por otra parte observamos que el comportamiento de las células derivadas de EDL y sóleo *in vitro* es muy similar, por lo que deducimos que la mayor susceptibilidad *in vivo* de las unidades motoras "fast" frente a las "slow" es causada por las distintas características de las dos fibras musculares y/o de las motoneuronas que las inervan más que de diferencias en las células satélite de cada tipo de fibra. Estos resultados respondieron a las cuestiones planteadas hasta ese momento, sin embargo la temprana alteración de las células madre derivadas del músculo de los animales SOD1-G93A de 40 días, cuando hay autores que describen los primeros signos musculares, nos llevó a preguntarnos si este comportamiento también se remonta a las primeras fases postnatales del individuo, cuando no existe ninguna pérdida descrita de conexiones neuromusculares, y el tiempo de acción de la enzima mutada sobre las células es bastante menor que en los estadios estudiados hasta el momento. Esta cuestión nos llevó a desarrollar el trabajo que recoge el manuscrito 6.

2.2.5.6. Manuscrito 6 Alteración del programa miogénico en ratones neonatos modelo de ELA SOD1-G93A

El objetivo fue valorar si la reducción en la capacidad de proliferación de las células en los cultivos de células satélite de animales SOD1-G93A se mantenía desde los primeros estadios postnatales cuando no existen evidencias de denervación y el periodo de acción de la enzima mutada es reducido. Para responder a esta cuestión se desarrollo un planteamiento experimental similar al presentado en el manuscrito 5 pero en ratones neonatos de entre 7 y 10 días. Debido a su corta edad, además de genotipar los animales fue necesario sexarlos ya que en este estudio se consideró interesante incluir ratones modelo SOD1-G93A de ambos sexos y de los cuales se extrajeron los músculos extensor digital largo (EDL) y sóleo (SOL) que habían sido objeto de interés en los anteriores trabajos (ver manuscrito 2). Además de la determinación de la capacidad de proliferación desde los 3.5 hasta los 7.5 días de cultivo, y la cuantificación de la expresión génica de *Pax7* y *Myod1* (a día 5.5 de cultivo) y miogenina (a día 7.5 de cultivo), en estos mismos puntos se procedió al marcaje inmunocitoquímico de las células para la detección de esas mismas proteínas y se realizó un recuento del número de células positivas para cada anticuerpo en los cultivos de cada grupo. De esta forma se observó la capacidad de expresión proteínica de estos factores en la células *in vitro*. Los resultados revelaron una menor capacidad de proliferación de las células satélite de los animales SOD1-G93A *in vitro*; esta tendencia, que no siempre alcanzó la significación estadística, coincidió en los donantes de ambos sexos y en los dos músculos estudiados. A nivel molecular, cabe destacar la perfecta correlación encontrada entre los niveles proteínicos y de transcritos a diferencia de lo observado *in vivo* en fases más avanzadas de la enfermedad (manuscrito 1). Esto nos hace suponer que las discrepancias observadas *in vivo* en fases más avanzadas de la enfermedad, son consecuencia del tiempo de acción de la enzima mutada sobre las células satélite, y que en neonatos es insuficiente para alterar los mecanismos de traducción y regulación proteínica, o a efectos de factores externos a la propia célula. En los cultivos derivados de donantes macho SOD1-G93A la disminución de la proliferación coincidió con el descenso de los transcritos y la proteína de *Pax7*, *Myod1* y miogenina. Sin embargo, en los cultivos de ratones SOD1-G93A derivados de hembras, a pesar de observarse una disminución de la capacidad proliferativa de sus células satélite, a nivel molecular, contrariamente a lo esperado, se observó una inducción de los factores indicadores de activación (*Myod1*) y diferenciación (miogenina) tanto a nivel génico como proteínico. En este sentido,

se ha descrito que el estrés oxidativo inhibe la expresión de los factores de regulación miogénica, y que las células de las hembras, ya desde el periodo embrionario tienen mayor resistencia a este daño oxidativo, lo que explicaría el incremento observado a nivel de transcritos y proteínico en las hembras y no en los machos. Por otra parte, independientemente de su respuesta a nivel molecular, las células satélite de ambos sexos mostraron una menor capacidad de proliferación, lo que puede ser consecuencia de la formación de agregados de la propia enzima, y/o la interacción física de éstos con proteínas reguladoras del ciclo celular o la alteración de la función mitocondrial y reticular, lo que interferiría con la capacidad de proliferación. Finalmente, hemos apreciado también que la proliferación y expresión de factores miogénicos de las células satélite de ambos músculos es idéntico, lo que coincide con los resultados obtenidos en fases posteriores de la enfermedad (manuscrito 5), y corrobora nuestra hipótesis de que la mayor selectividad de la enfermedad sobre las unidades motoras "fast" deriva de las distintas propiedades de los músculos y las motoneuronas que los inervan más que de una intrínseca vulnerabilidad de sus células satélite.

En conjunto estos trabajos pretenden caracterizar el estado de las células satélite musculares en el modelo murino de ELA SOD1-G93A desde los primeros momentos de la vida del animal hasta las fases terminales de la enfermedad. Hemos cuantificado el reservorio de dichas células y determinado su capacidad de activación y de proliferación *in vivo* e *in vitro*. Asimismo hemos determinado el estado de los factores que regulan el proceso de regeneración inducido por dichas células. Finalmente hemos delimitado la influencia de variables como la edad, el sexo o el tipo de fibra muscular en estos parámetros tanto en animales SOD1-G93A como controles no transgénicos.



PUBLICACIONES

3. PUBLICACIONES

3.1. Neurodegenerative Diseases, 2011

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Manzano R, Toivonen JM, Oliván S, Calvo AC, Moreno-Igoa M, Muñoz MJ, Zaragoza P, García-Redondo A, Osta R.

Neurodegener Dis. 2011;8(5):386-96

Modeling Neurodegenerative Diseases in vivo

Neurodegenerative Diseases

Neurodegenerative Dis 2011;8:386–396
DOI: 10.1159/000324159

Received: July 22, 2010
Accepted after revision: January 5, 2011
Published online: February 23, 2011

Altered Expression of Myogenic Regulatory Factors in the Mouse Model of Amyotrophic Lateral Sclerosis

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Key Words
Amyotrophic lateral sclerosis · Motor neuron disease · Myogenic regulatory factor · Muscle · Denervation

Abstract
Background: In the superoxide dismutase 1 (SOD1)-G93A mouse model of amyotrophic lateral sclerosis (ALS), skeletal muscle is a key target of mutant SOD1 toxicity. However, the expression of factors that control the regenerative potential of the muscle is unknown in this model. ***Objective:*** To characterize the expression of satellite cell marker *Pax7* and myogenic regulatory factors (MRF) in skeletal muscle of SOD1-G93A mice at different stages of the disease. ***Methods:*** The expressions of *Pax7, Myod1, Myf5* and myogenin *(Myog)* were determined by quantitative real-time PCR and by Western blotting from the grouped gastrocnemius, quadriceps and soleus muscles of SOD1-G93A mice at presymptomatic, symptomatic and terminal stages of the disease, and from surgically denervated wild-type gastrocnemius muscles. ***Results:*** *Pax7* mRNA and MYF5 protein were upregulated in presymptomatic mice, coinciding with increased muscle damage marker *Rrad* and chemokine *Ccl5*. All MRF transcripts and most proteins (excluding MYOG) were increased, starting from 3 months of age, simultaneously with increased expression of denervation marker Chrna1. However, in the terminal stage, no protein increase was evident for *Pax7* or any of the MRF despite the increased mRNA levels. The transcripts for chemokine *Ccl2* and chemokine receptor *Cxcr4* were increased starting from the onset of symptoms. ***Conclusions:*** The characterization of *Pax7* and MRF in SOD1-G93A mice reveals a progressive induction of the myogenic program at the RNA level, but a blunted protein level response at late stages of the disease. Altered posttranscriptional and posttranslational mechanisms likely to operate, as well as the potential role of chemokine signaling in mutant SOD1 muscle, are discussed.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic motor neuron (MN) disease characterized by progressive muscle weakness caused by gradual degeneration of upper and lower motor neurons [1]. Approximately 10% of ALS cases are familial (FALS), and one fifth of these are caused by mutations in the gene encoding antioxidant enzyme Cu/Zn superoxide dismutase 1 (SOD1) [2]. This suggests that oxidative stress is one of the key mecha-

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nisms that underlie ALS pathogenesis. Transgenic mouse models of FALS have been instrumental in shedding light on pathophysiological and molecular aberrations in human ALS patients [3]. The most frequently used model of FALS is a ubiquitous overexpressor of human SOD1 that carries a glycine-to-alanine substitution at residue 93 (SOD1-G93A) [4] and results in toxic gain of SOD1 function [5]. Although the precise cellular mechanisms of mutant SOD1 (mSOD1) toxicity remain unknown, SOD1-G93A mice develop pathological and clinical features closely resembling human ALS [6]. The primary trigger for the pathogenic events is thought to be the interruption of the nerve connection at the neuromuscular junction (NMJ), which precedes axonal degeneration and death of MN cell bodies [7–10]. Debate still continues as to what extent the muscle denervation in ALS models is of neuronal origin: neuron-restricted expression of the mSOD1 does not replicate the hallmarks of the disease [11, 12], although this may depend on the mSOD1 expression level [13]. On the other hand, the non-cell-autonomous contribution to MN degeneration by non-neural cells (such as microglia, astrocytes and muscle) is evident [14], and suggests that alterations in multiple cell types act synergistically to exacerbate the disease.

Involvement of skeletal muscle in ALS pathophysiology was first inferred from early muscle hypermetabolism in mSOD1 mice and in human patients [15–17]. Experimentally induced muscle hypermetabolism dismantles NMJ in wild-type mice as well as it promotes disease progression in mSOD1 animals [18]. Additionally, a denervation-promoting factor, Nogo-A, is upregulated in ALS skeletal muscle [19, 20]. Recent studies have demonstrated that skeletal muscle-specific mSOD1 overexpression is sufficient to induce oxidative damage and trigger ALS symptoms [21] as well as MN degeneration [22]. Collectively, these studies suggest that early changes in ALS muscle may promote denervation.

Postmitotic skeletal muscle fibers show a great degree of plasticity in response to exercise, damage and disease [23]. The capacity for tissue repair derives largely from activation of satellite cells (SC), mononuclear stem cells that reside beneath the basal membrane of the mature muscle fiber [24, 25]. *Pax7* is a paired-box transcription factor essential for the survival, maintenance and myogenic potential of SC [26, 27]. Muscle growth and differentiation are regulated by a complex interaction between myogenic regulatory factors (MRF) such as *Myf5, Myod1* and myogenin *(Myog),* which specifically activate the production of key muscle genes [28]. Following muscle injury, quiescent *Pax7*-positive SC migrate to the site of damage, upregulate the myogenic determinants *Myf5* and *Myod1* and reenter the cell cycle [29–31]. These activated myoblasts subsequently downregulate *Pax7* and induce *Myog* to initiate terminal differentiation. This is followed by fusion with other SC or with damaged myofibers, cell cycle exit and progressive loss of MRF expression.

Transcriptional induction of MRF upon surgical denervation is well described [32–36]. However, although the denervation of NMJ in ALS is evident, potential alterations in myogenic regulation in FALS models remain unexplored. Skeletal muscle tissue in mSOD1 mice is not only affected by denervation but also suffers from nerve-independent loss of muscle homeostasis [21, 37]. Muscle-autonomous mSOD1 toxicity may limit the capacity of mutant muscle to regenerate, and altered expression of SC markers (such as *Pax7*) or MRF could serve as early indicators of such a process. Muscle regeneration may also be affected by recruitment of muscle-resident or circulating myogenic progenitor cells other than SC [38–42]. Directional cell migration to muscle may be enhanced by the action of secreted chemotactic cytokines (chemokines), some of which are upregulated at least in the circulation and the cerebrospinal compartment of ALS patients [43–49]. Intriguingly, many chemokines and their receptors are increased during regenerative myogenesis in vivo, and some have been shown to stimulate myoblast migratory activity [50, 51]. However, chemokine expression in ALS muscle remains unexplored.

Clinical and pathological similarities between sporadic and inherited ALS have created the anticipation that models of FALS may provide insight into mechanisms of both forms of the disease and, ultimately, contribute to therapeutic strategies in humans. Skeletal muscle is regarded as one of the most promising targets for therapeutic interventions including molecular therapy, although considerable obstacles remain to be overcome as well [23]. To elucidate potential alterations to myogenic factors in mSOD1-expressing muscle, we systematically investigated transcript and protein level changes in *Pax7* and MRF at different ages of SOD1-G93A mice. Additionally, we looked for transcriptional alterations in muscle chemokine signaling, which may provide alternative cues for myogenic precursor recruitment in ALS.

Materials and Methods

All experimental procedures were approved by the ethics committee of the institution and followed the international guidelines for the use of laboratory animals. The transgenic mice B6SJL-Tg(SOD1-G93A)1Gur/J [4] expressing a high copy number of the

G93A mutant form of human SOD1 were purchased from The Jackson Laboratory (Bar Harbor, Me., USA) and were housed under a 12-hour light/12-hour dark cycle at 21–23°C with a relative humidity of 55%. Food and water were available ad libitum. The transgenic colony was maintained by breeding hemizygous SOD1-G93A males with B6SJL wild-type female littermates. The genotypes were determined from tail samples as described in the Jackson Laboratory protocol. Heterozygous male mice and their nontransgenic male littermates were used for all experiments.

Surgical Denervation

Six male wild-type (nontransgenic) mice of the B6CLJ strain were anesthetized (pentobarbital 30 mg·kg^{-1}, i.p.) at the age of 60 days, and muscle denervation was performed unilaterally in the right hindlimb by extracting a 5-mm segment of the sciatic nerve through an incision in the mid-posterolateral area of the thigh. The left limb was left intact and served as a control. The incision was closed with silk sutures and washed daily with antibacterial solution to prevent infection. The absence of the toe-spreading reflex was confirmed daily. Two weeks after surgical denervation (at the age of 74 days), the animals were sacrificed by cervical dislocation and the gastrocnemius muscles were dissected and frozen immediately in liquid nitrogen.

Extraction of Muscle Samples

Hemizygous SOD1-G93A males and age-matched nontransgenic wild-type control males were sacrificed by cervical dislocation at postnatal (P) age of P40, P60, P90 or P120 days. At each age, 5 animals per genotype were used. Quadriceps, gastrocnemius and soleus muscles from both hindlimbs were individually dissected, snap frozen in liquid nitrogen, and then temporarily stored at −70°C. The quadriceps muscle group (vastus lateralis, vastus medialis and vastus intermedius) and gastrocnemius muscles (medial and lateral) both consist of approximately 60% fast-fatigable (FF) fibers, 35% fast fatigue-resistant fibers and 5% slow (S) fibers, whereas the soleus is composed of 58% S fibers and 42% fast fatigue-resistant fibers [52]. This group of muscles was selected to ensure enough material for both RNA and protein studies, and because it consists of muscles with a high percentage of FF fibers or S fibers, which are most vulnerable or resistant to denervation in the SOD1-G93A model, respectively [8–10]. The dissected muscles from each mouse were pooled and pulverized in a cold mortar with liquid nitrogen. The powdered tissue was divided equally in two prechilled tubes, one for RNA extraction and the other one for protein extraction.

Quantification of mRNA Expression

For total RNA extraction, powdered muscle tissue was further homogenized using a PRO200 homogenizer (PRO Scientific Inc.) and then processed according to the TRIzol Reagent protocol (Invitrogen). The RNA samples were processed with the Turbo DNA-free kit (Ambion) to eliminate genomic DNA. Reverse transcription was carried out according to the SuperScript™ First-Strand Synthesis System kit (Invitrogen) using random hexamers with 1 μg of template RNA in a final volume of 20 μl. TaqMan® (Applied Biosystems) probe-based quantitative PCR reactions were performed from 1 μl of 1:10 diluted cDNA in a reaction mixture containing 1× TaqMan Universal PCR Master Mix (No AmpErase® UNG) and 1× TaqMan primer/probe mix in a final volume of 10 μl. Triplicate reactions from each cDNA were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following thermal cycling parameters: incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reaction efficiencies of the primer/probe sets approached 100% (see online supplementary table 1, www.karger.com/doi/10.1159/000324159). In the same conditions, reference gene amplification was performed using TaqMan primer/probe mixtures for three reference genes (18S rRNA, Gapdh and β-actin). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, where the data are presented as a fold change in gene expression normalized to the reference genes and relative to the nontransgenic age-matched calibrator [53] (online suppl. materials and methods). Analysis of *Ccl5* (Mm01302428 m1), *Ccl2* (Mm00441242_m1) and *Cxcr4* (Mm01292123_m1) transcript levels was carried out in identical reaction conditions but using Applied Biosystems Custom TaqMan Microfluidic Cards and 7900HT Fast Real-Time PCR Systems. Data processing and analysis were carried out as detailed above for the individual reaction assays.

Western Blot Analysis

The powdered muscle tissue was suspended in ice-cold buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin/aprotinin and 1 μg/ml pepstatin. The samples were centrifuged at 3,000 rpm at 4°C for 10 min, and the total protein in the supernatant fractions was quantified by the BCA method (Sigma-Aldrich). The proteins (60 μg per well) were separated by SDS-PAGE alongside molecular weight standards, and subsequently transferred to polyvinylidene fluoride membranes (GE Healthcare) at 100 V for 1 h in a transfer buffer containing 25 mM Tris, 200 mM glycine and 10% (v/v) methanol. The membranes were blocked in Tris-buffered saline supplemented with 0.1% Tween 20 and 5% (w/v) powdered skim milk for 1 h at room temperature and incubated overnight at 4°C with a primary antibody diluted in a freshly prepared blocking buffer. The antibody dilutions used were: PAX7 (Ab34360; Abcam) 1:250; MYOD1 (sc-304; Santa Cruz Biotechnology Inc.) 1:300; MYOG (sc-576; Santa Cruz Biotechnology) 1 μg/ml; MYF5 (sc-302; Santa Cruz Biotechnology 1:200), and GAPDH (sc-25778; Santa Cruz Biotechnology) 1:5,000. Subsequently, the membranes were incubated for 1 h at room temperature in a solution containing the horseradish peroxidase-conjugated secondary antibody (sc-2004; Santa Cruz Biotechnology), diluted 1:5,000 in blocking buffer. Several washes with Tris-buffered saline and 0.1% Tween 20 were performed after incubation with primary and secondary antibodies. Blots were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and were exposed to Curix RP2 Plus X-ray film (Agfa). The computer-assisted intensity analysis of the detected bands was performed with the AlphaEaseFC software (Bonsai Technologies Group). The mean value of normalized intensities from SOD1-G93A animals was compared to that of the wild-type mice to obtain a fold change. For standard errors of the mean, each normalized SOD1-G93A value was compared to the normalized mean intensity of the wild type.

Statistics

Statistics were performed using Statistica 5.0 (Statsoft). Comparisons were carried out by the Student t test, and $p < 0.05$ was considered significant.

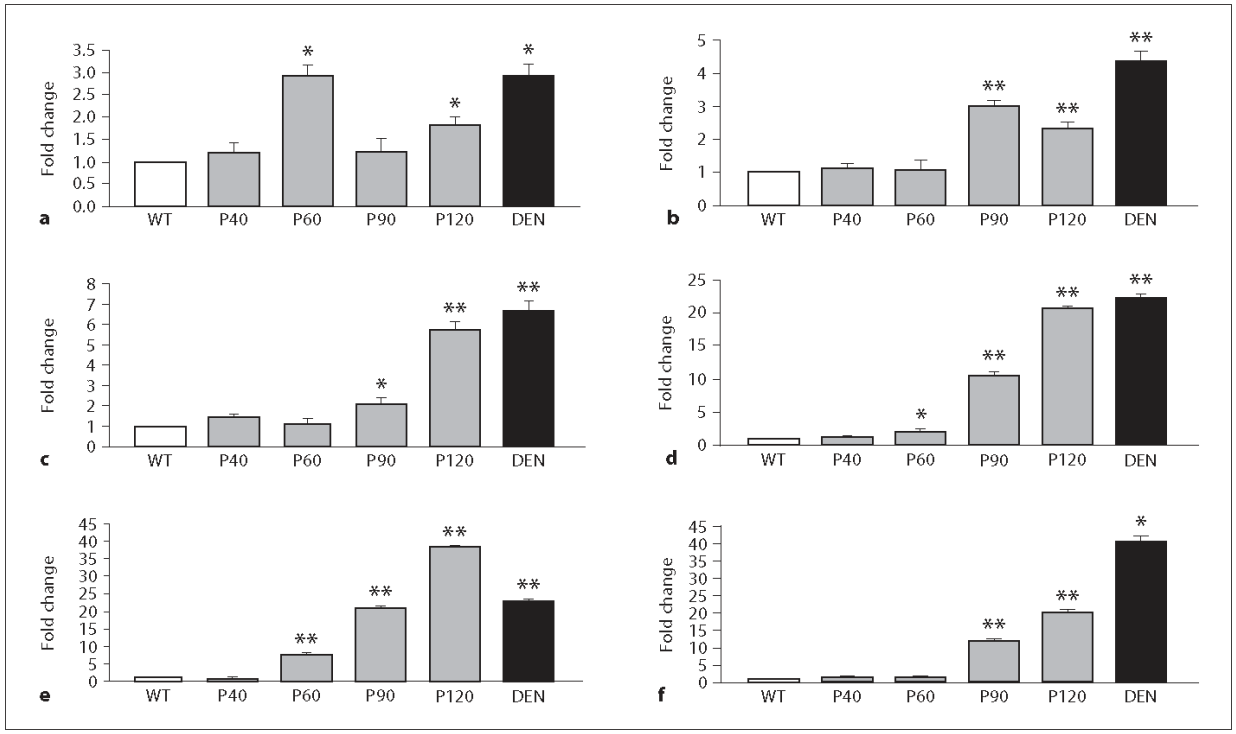

Fig. 1. Upregulation of *Pax7*, MRF, *Rrad* and *Chrna1* transcripts in SOD1-G93A and denervated muscles. The results are shown as fold change in SOD1-G93A animals (grey bars) relative to wild-type littermates (WT; white bars) at each indicated age (P40, P60, P90 and P120), or relative to the nonoperated limb in denervated mice (DEN; black bars). Error bars: SEM. * $p < 0.05$; ** $p < 0.001$. **a** *Pax7.* **b** *Myf5.* **c** *Myod1.* **d** *Myog.* **e** *Rrad.* **f** *Chrna1.*

Results

Transcriptional Upregulation of Pax7 and MRF in mSOD1 Muscles

To gain insight into the regulation of the myogenic process in SOD1-G93A mice at different stages of the disease, quantitative RT-PCR was performed at P40, P60, P90 and P120. These time points were selected to monitor changes at early (P40) and late presymptomatic (P60) stages, as well as at the onset of the symptomatic (P90) and terminal stages (P120). Relative expression (fold difference) of *Pax7, Myf5, Myod1* and *Myog* (fig. 1a–d) was determined for each age, using RNA derived from pooled quadriceps, gastrocnemius and soleus muscles of SOD1-G93A hemizygotes and their age-matched nontransgenic littermates. In mSOD1-expressing muscle, the production of reactive oxygen species (ROS) is elevated, which in turn activates the expression of *Rrad* (Ras-related associated with diabetes), an early marker in ALS patients and mSOD1 mice [37]. Although *Rrad* is also upregulated by denervation, its transcription precedes that of the 'ROS-insensitive' denervation marker *Chrna1* (cholinergic receptor, nicotinic, alpha) [37]. Monitoring of *Rrad* (fig. 1e) enabled us also to estimate the first appearance of the muscle damage before the onset of symptoms. Similarly, the extent of denervation was monitored by induction of *Chrna1* (fig. 1f) as this receptor is transcriptionally regulated by electrical activity [32, 34, 54] and not by ROS [37]. To compare the effects of surgical axotomy with those caused by SOD1-G93A mutation, we also performed sciatic denervation on wild-type mice at P60 and analyzed the expression of the above-mentioned factors in the gastrocnemius muscles at P74, 2 weeks after the operation (fig. 1a–f, black bars).

At the early presymptomatic stage of the SOD1-G93A mice (P40), none of the genes under study were significantly affected (fig. 1a–f). Unaltered expression of *Rrad* and *Chrna1* at this time point indicates that muscle dam-

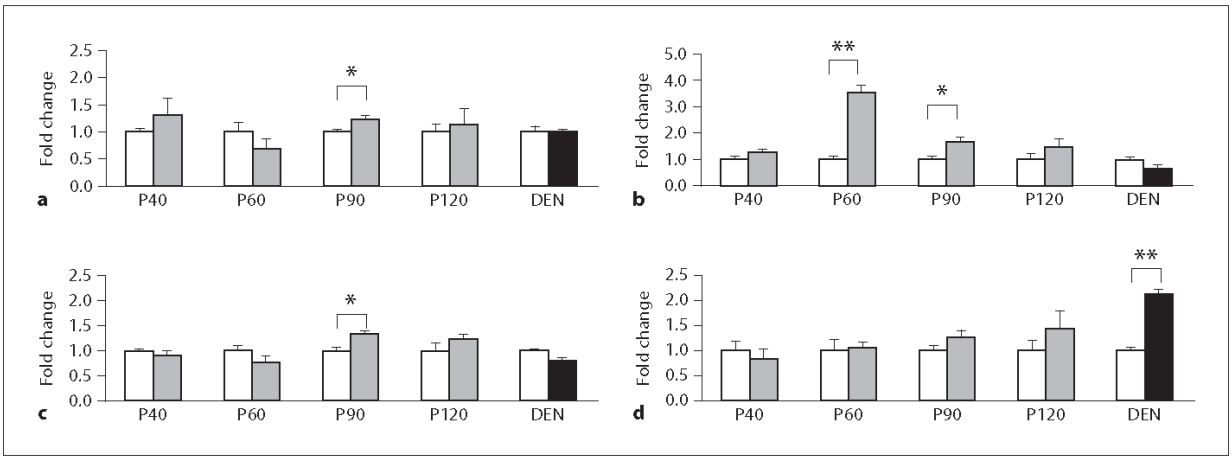

Fig. 2. Protein level regulation of PAX7 and MRF in SOD1-G93A and denervated muscles. The results are shown as fold change in SOD1-G93A animals (grey bars) relative to wild-type littermates (white bars) at each indicated age (P40, P60, P90 and P120), or relative to the nonoperated limb in denervated mice (DEN; black bars). Error bars: SEM. * $p < 0.05$; ** $p < 0.001$. Representative Western blot membranes are shown in online supplementary figure 1. **a** PAX7. **b** MYF5. **c** MYOD1. **d** MYOG.

age (and associated ROS production) and denervation in SOD1-G93A animals either has not commenced or is minimal, which is consistent with previously reported data [10, 37]. At the later presymptomatic stage (P60), *Pax7* transcript levels were 3-fold higher than in the nontransgenic controls (fig. 1a). However, *Myf5* (fig. 1b) or *Myod1* (fig. 1c) were not affected, whereas *Myog* (fig. 1d) and *Rrad* (fig. 1e) – but not *Chrna1* (fig. 1f) – were modestly upregulated. At the onset of ALS-like symptoms in the SOD1-G93A animals (P90), a 3-fold upregulation of *Myf5* (fig. 1b), a 2.1-fold upregulation of *Myod1* (fig. 1c) and a robust 10.6-fold upregulation of *Myog* (fig. 1d) was observed, whereas *Pax7* mRNA (fig. 1a) was not significantly affected at this stage. Also, a 11.6-fold induction of *Chrna1* (fig. 1f) was first seen at this age, where the loss of NMJ is already significant. At the onset of the terminal stage (P120), all genes studied were upregulated, ranging from 1.8-fold for *Pax7,* 2.3-fold for *Myf5,* 6-fold for *Myod1* to 20-fold for *Myog.* All genes studied were also upregulated in the denervated gastrocnemius muscles of the nontransgenic wild-type mice compared with the intact control muscles from the same animals (fig. 1a–f, black bars). These observations provide the first evidence of a systematic transcriptional activation of the myogenic program in SOD1-G93A animals and suggest a great degree of similarity in the regulation of MRF mRNA in denervated and mSOD1 muscles. However, as observed earlier in mouse models of ALS [37], *Rrad* was upregulated earlier than *Chrna1,* suggesting that mSOD1-mediated oxidative stress in the muscle temporally precedes that caused by denervation.

Blunted Increment of Pax7 and MRF Proteins in mSOD1 Muscles

Induction of myogenic factor mRNA in SOD1-G93A mice prompted us to investigate whether the increase in Pax7 and MRF transcripts was also reflected in the protein level. Western blot analysis was carried out on the same powdered muscle samples as for the mRNA analysis to eliminate possible individual variation between the animals (see Materials and Methods section). At the early presymptomatic stage (P40), the proteins studied were not significantly altered (fig. 2a–d). After this stage, a rather different picture emerged from the protein level studies of PAX7 and MRF as compared with the transcript data. Despite a significant upregulation of *Pax7* (and to a lesser extent *Myog*) mRNA at P60, the corresponding protein levels were not increased (fig. 2a, d). In contrast, MYF5 was 3.5-fold upregulated (fig. 2b) despite unaltered *Myf5* mRNA levels at this stage. At P90, PAX7 (fig. 2a), MYF5 (fig. 2b) and MYOD1 (fig. 2c) proteins were modestly but significantly upregulated, whereas the increment in MYOG was suggestive but not significant (fig. 2d). Most surprisingly, the uniform increase in MRF mRNA levels at P120 was not reflected at the protein level despite the 2.5-fold, 6-fold and 20-fold increases in

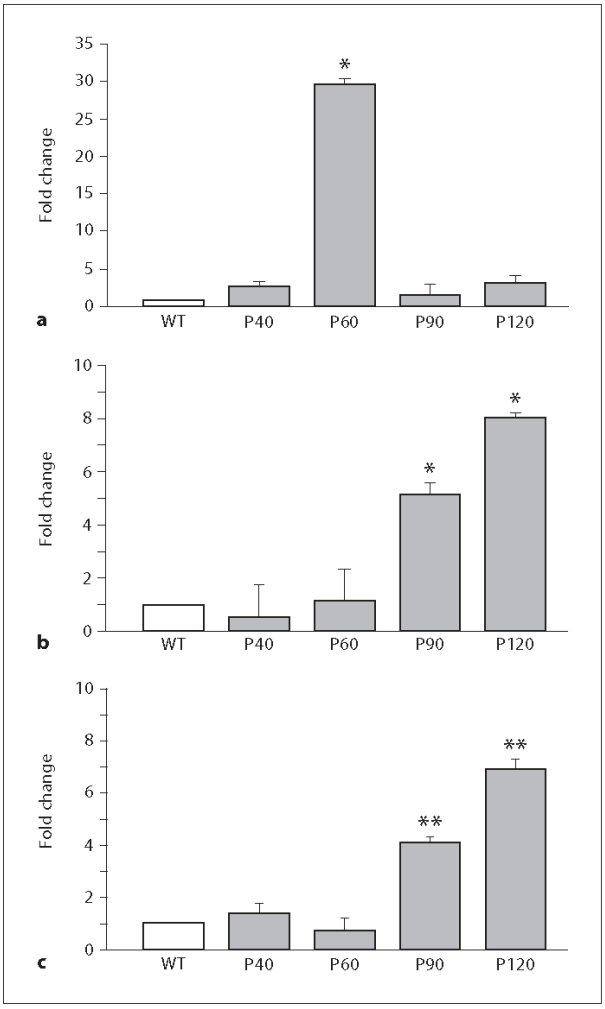

Fig. 3. Altered levels of *Ccl5* (**a**), *Ccl2* (**b**) and *Cxcr4* (**c**) transcripts in SOD1-G93A muscles. The results are shown as fold change in SOD1-G93A animals (grey bars) relative to wild-type littermates (WT; white bars) at each indicated age (P40, P60, P90 and P120). Error bars: SEM. * $p < 0.05$; ** $p < 0.001$.

Myf5, *Myod1* and *Myog* mRNA, respectively. Although MYOG protein showed a tendency to increased relative abundance at advanced stages of the disease, the changes were not significant for any age. In denervated mice (fig. 2a–d, black bars), PAX7, MYF5 and MYOD1 proteins were not significantly altered despite increased mRNA levels. However, MYOG, which was transcriptionally upregulated in denervated muscle, also showed a 2-fold increase in protein level upon denervation.

These observations reveal both similarities and differences between denervated and SOD1-G93A muscles. PAX7, MYF5 and MYOD1 protein levels were increased at P90 in SOD1-G93A muscles, whereas this was not observed in nontransgenic mice that had been denervated for 2 weeks. The opposite was true for MYOG protein, which was not significantly increased in the mSOD1 muscles at any point but was clearly upregulated upon denervation. Therefore, although transcriptional changes in denervated and mSOD1 muscles are largely consistent (all upregulated), the MYOG protein induction (or lack of it) suggests that differences may exist between SOD1-G93A and denervated muscles.

Altered Expression of Ccl5, Ccl2 and Cxcr4 in SOD1-G93A Muscle

It is intriguing that *Pax7* mRNA was increased in SOD1-G93A muscles already at 60 days of age (fig. 1a). This increase was not likely to be denervation induced because MRF or *Chrna1* mRNA, which respond transcriptionally to surgical denervation (fig. 1, black bars), were not altered at this stage. This led us to investigate an alternative explanation for the origin of the early increment in *Pax7* mRNA expression. Because the *Ccl5* chemokine is upregulated in ALS patients [47] and has the potential to stimulate myoblast migratory activity [50], it was regarded as a promising candidate for the recruitment of cells with myogenic potential by the injured mSOD1 muscle. Compared to the age-matched wild-type muscle, *Ccl5* mRNA was 29-fold elevated in SOD1-G93A muscles at P60 (fig. 3a), which coincided with a 3-fold increase in *Pax7* transcripts (fig. 1a). At this stage, the expression of *Rrad* (fig. 1e) was 8-fold higher in SOD1-G93A animals, revealing the commencing muscle damage. These results support the hypothesis that presymptomatic mSOD1-mediated toxicity may cause the recruitment of circulating myogenic cells to the muscle, which may then partly contribute to the *Pax7*-expressing muscle progenitor pool. To further investigate chemokine-mediated signaling in muscle tissue of SOD1-G93A animals, we looked for mRNA expression of another chemokine, *Ccl2*, which is known to be upregulated in ALS [48, 49] and during in vitro myogenesis [51]. Additionally, we examined the mRNA expression of *Cxcr4*, a chemokine receptor that – together with its ligand, stromal cell-derived factor 1 (SDF-1) – is known to mediate the migration and fusion of muscle cells in vitro and may regulate adult regenerative myogenesis [51]. Unlike *Ccl5*, levels of which were relatively higher in SOD1-G93A animals only at presymptomatic

stage P60, both *Ccl2* (fig. 3b) and *Cxcr4* (fig. 3c) were increased at late stages, starting from the symptomatic stage. These results suggest that, as observed in other tissues of ALS patients, the chemokines *Ccl5* and *Ccl2* are affected in the mSOD1 muscle, although their increased expression is observed at different stages of the disease. Additionally, the results implicate the Cxcr4-SDF-1 axis as a potential contributor to SOD1-G93A disease progression.

Discussion

A direct influence of skeletal muscle tissue on pathogenesis in the mouse models of ALS has recently been demonstrated [21, 22]. Here, we investigated the expression of primary determinants of the regenerative potential of the muscle, i.e. SC function *(Pax7),* myoblast proliferation *(Myf5, Myod1)* and differentiation *(Myog)* in the SOD1-G93A model of ALS. A gross increment in MRF species was evident at the symptomatic stage (P90), later than the upregulation of *Rrad* and in parallel with the induction of denervation marker *Chrna1.* The drastic increase in MRF transcripts in terminal (P120) SOD1-G93A animals did not result in increased levels of MRF proteins, suggesting that the attempted regeneration of SOD1-G93A muscle by MRF transcript upregulation is futile. Presymptomatic increase in *Pax7* mRNA coincided with the upregulation of chemokine *Ccl5.* Furthermore, we demonstrated an increased mRNA expression of chemokine *Ccl2* and chemokine receptor *Cxcr4* in SOD1-G93A muscle at later disease stages, which mirrored those of the MRF transcripts. In the following discussion, we will first highlight the similarities and differences between SOD1-G93A and denervated muscle. Then, we will discuss the levels of molecular regulation and posttranscriptional mechanisms that may underline the discrepancies in MRF transcript and protein levels and potentially exacerbate the ALS-associated muscle atrophy. Finally, we will discuss how the increased chemokine expression in mSOD1 muscle may contribute to the availability of myogenic precursors in the mutant muscle.

MRF transcripts, and occasionally proteins, have been shown to be upregulated in response to denervation [32–36, 55–58]. Here, the upregulation of MRF transcripts in muscles from denervated limb and their increased relative abundance in SOD1-G93A muscles towards the end of the disease paralleled that of *Chrna1,* suggesting that increments in *Myf5, Myod1* and *Myog* transcripts are mainly due to decreased electrical stimulation caused by denervation. Based on proportional muscle mass in the pooled gastrocnemius, quadriceps and soleus muscles, the contribution of FF fibers was expected to be close to 60%, although this may have shifted towards more fatigue-resistant types in older SOD1-G93A mice [59]. Therefore, rapid transcriptional induction of MRF probably indicates changes in FF fibers that are innervated by the most vulnerable motor axons in mSOD1 mice [9, 10, 60]. The MRF studied (but not SC-specific *Pax7*) are also known to be expressed both in activated myoblasts and in mature myofibers upon denervation [55, 57, 61]. How the described increase in MRF in mSOD1 muscles reflects transcription in SC versus myofibers remains currently unknown.

After 2-week denervation, the MYOG protein level was upregulated in wild-type mice, whereas PAX7, MYF5 and MYOD1 were unaffected. A postdenervation period of 2 weeks was tested here to evaluate the effects of moderately long-term denervation, which we reasoned would better compare with the situation in SOD1-G93A animals. Because MRF expression is strongly induced within days after sciatic denervation and decreases thereafter [32, 34, 35], it is possible that the time point used here was too late to detect increased MYF5 and MYOD1 proteins. Discrepancies between MRF transcript and protein levels have also been reported earlier [62, 63]. Because of a general lack of simultaneous RNA/protein level studies and a wide variety of techniques and experimental species used, a universally valid MRF response to denervation cannot be stated [64]. However, the fact that *Myog* transcripts were equally upregulated in denervated and SOD1-G93A muscle, but the MYOG protein increase was only apparent upon denervation, suggests that posttranscriptional regulation of this factor in SOD1-G93A mice may be altered.

Several molecular and pathophysiological alterations may contribute to the discordance between MRF mRNA and protein levels in SOD1-G93A mice. Although MRF proteins were not upregulated at the terminal stage, they were not depleted either (fig. 2). MRF can increase their own transcription and cross-regulate one another's expression [65]. The transcription factor activity of MYOD and MYOG is regulated by phosphorylation [66, 67], relies on hetero-oligomerization with E-proteins [68], and is inhibited by Id1, a negative regulator of MRF/E-protein oligomerization [69]. Therefore, elevated transcription of MRF mRNA in SOD1-G93A muscles could potentially result from the altered phosphorylation status of the MRF proteins or, alternatively, from the changed balance be-

tween their oligomerization partners and inhibitors, such as E-proteins and Id1.

Skeletal muscle atrophy induced by pathological conditions involving oxidative damage is characterized by increased proteolysis via the ubiquitin-proteasome pathway [70, 71]. Consistently, the expression of ubiquitin ligases is elevated in human ALS patients and mSOD1 mice [72]. Both MYOD1 and MYOG are targets for proteasomal activity [73, 74], and ubiquitin-proteasome-mediated degradation of MYOD1 occurs in several models of skeletal muscle atrophy [75]. Interestingly, ubiquitin ligases in denervated muscle are under direct transcriptional control of MYOG, which therefore may promote the loss of muscle mass as well as differentiation [76]. Muscle-specific expression of insulin-like growth factor 1 attenuates muscle wasting and reduces protein ubiquitination [77], which is associated with increased PAX7 and MYOG protein expression at the terminal stage of SOD1-G93A mice [78]. Therefore, insulin-like growth factor 1 may alleviate the metabolic processes that lead to the blunted protein level expression of these factors in SOD1-G93A muscle. Genetic or pharmaceutical targeting of the proteasome or associated enzymes could possibly serve as a candidate strategy to alleviate SOD1-G93A muscle pathology.

Rather than indicating direct transcriptional activation, increased MRF transcript abundance also derives from posttranscriptional mRNA stabilization. Both *Myod1* and *Myog* transcripts are targets of the mRNA-stabilizing enzyme HuR, which is elevated in regenerating myofibers in vivo [79]. Under endoplasmic reticulum (ER) stress, global changes in transcript abundance are largely controlled by mRNA stability. Although a possible contribution of ER stress to the pathobiology of the ALS muscle remains unexplored, it has been shown to take place in affected neurons of human ALS patients and mSOD1 models [80]. Equally, RNA oxidation occurs in neurons of both ALS patients and mice overexpressing mSOD1 [81]. Because mRNA stabilization under ER stress [82] and mRNA oxidation in general [83] are associated with decreased protein translation, they may have a role in the observed disparity between the MRF transcripts and their corresponding proteins in SOD1-G93A muscle.

Besides the regulation of MRF at the late disease stages, a discordance was observed between *Pax7* and *Myf5* transcript and protein levels at the presymptomatic stage. *Pax7* mRNA but not protein was increased at P60, whereas *Myf5* was only increased in protein level (fig. 1, 2). This inconsistency does not derive from muscle sample variation as the same frozen tissue was used for both RNA and protein experiments (see Materials and Methods section). Although further experiments are required to formally address these observations, they may also be linked. *Pax7* has recently been shown to be a target of the muscle-specific microRNA miR-1 and miR-206 [84], which are upregulated in mSOD1 mice [85]. Inhibition of these microRNA increases the PAX7 protein level in vivo and inhibits differentiation [84]. MYF5 potently upregulates these microRNA in developmental myogenesis [86] and may therefore inhibit *Pax7* mRNA translation. On the other hand, experiments on synchronized myoblasts have shown that MYF5 protein abundance is regulated by the cell cycle and that the variations in protein level are largely independent of those of *Myf5* mRNA, indicating posttranslational control [87, 88]. This property of *Myf5* regulation may contribute to the increased MYF5 protein level despite a lack of transcriptional increase at P60. More detailed analysis is warranted to test these hypotheses on mSOD1 muscle in vivo.

Within healthy skeletal muscle, the quiescent SC number largely remains constant. The relative increase in presymptomatic *Pax7* expression in SOD1-G93A muscle could indicate denervation-independent SC renewal/activation or, alternatively, recruitment of *Pax7*-expressing cells by muscle from other stem cell niches. Myogenic precursor recruitment could potentially be enhanced by chemokines such as *Ccl5,* which is upregulated in ALS patients [47]. *Ccl5* can stimulate myoblast migratory activity [50], and its transcripts were markedly increased at the presymptomatic stage in SOD1-G93A mice (fig. 3a). Increases at the symptomatic and terminal stages of the disease in chemokine *Ccl2* (fig. 3b), also increased in ALS patients [48, 49], and in chemokine receptor *Cxcr4* (fig. 3c) provide further support for altered chemokine-mediated signaling in SOD1-G93A muscles. *Cxcr4* is expressed in numerous adult stem cells [89], and its ligand, SDF-1, is known to be released from regenerating muscle to attract CXCR4-positive cells [90]. Although these findings are only correlative at this point, they encourage further experiments investigating the nature and regulation of potential myogenic precursor recruitment by chemokines in SOD1-G93A.

To conclude, we described the induction of the myogenic program in the course of the disease in SOD1-G93A mice and revealed both denervation-dependent and -independent effects on *Pax7* and MRF expression. These findings encourage further studies on the myogenic process in ALS and indicate that some caution may be required when data are interpreted solely based on tran-

script abundance. The blunted protein level accumulation of MRF described here should also guide future research in the evaluation of posttranscriptional and posttranslational mechanisms regulating myogenesis in mSOD1 muscles. Furthermore, research aiming at a detailed understanding of chemokine-mediated signaling in affected ALS muscles may help to improve the delivery of myogenic precursors to muscle tissue, which is of considerable interest to those working on therapeutic aspects of ALS.

Acknowledgments

We would like to thank Ana Pérez (CIMA, University of Navarra, Spain) for the MYF5 antibody. This study was supported by grants PI071133 and PI071283 from the National Health Institute Carlos III (ISCIII) of Spain, PIPAMER 08/08 and 09/09 from the Aragón Institute of Health Sciences, NDG07/07 from the Agencia Pedro Laín Entralgo Comunidad de Madrid, and the Project 'Tú eliges: tú decides' from Caja de Ahorros de Navarra in Spain.

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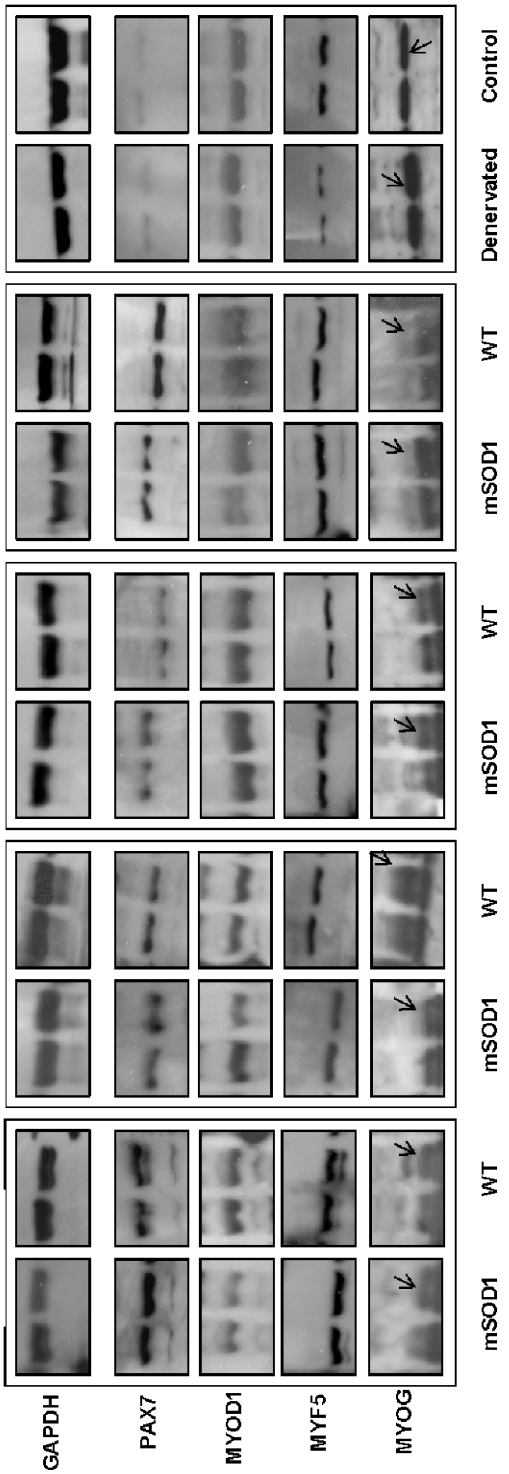
Supplementary Figure 1 (Manzano et al., 2010)



Supplementary Figure 1 Representative western blots from each antibody used. SDS-PAGE Separated protein extracts from two SOD1-G93A (mSOD1) and wild type (WT) non-transgenic littermates are show for each age. The arrow on MYOG blots indicated the 32 kDA band used to quantify MYOG levels (see Supplementary Materials and methods for details).

Supplementary Table 1.

Supplementary Table 1 The list and properties of TaqMan probes used for the quantitative gene expression studies.

Target	Assay ID[$]	Length*	$R^{2\wedge}$	Slope#	Ex[&]
Rn18S	4352930E	187 bp	1.0000	-3.4744	0.940
Gapdh	4352932E	107 bp	0.9777	-3.3763	0.978
Actb	4352933E	115 bp	0.9981	-3.4636	0.944
Pax7	Mm00834079_m1	64 bp	0.9986	-3.1442	1.079
Myod1	Mm00440387_m1	86 bp	0.9848	-3.2183	1.045
Myog	Mm00446194_m1	69 bp	0.9789	-3.1077	1.098
Myf5	Mm00435125_m1	71 bp	0.9927	-3.0299	1.138
Rrad	Mm00451053_m1	71 bp	0. 9938	-3.3963	0.970
Chrna1	Mm00431627_m1	67 bp	0.9951	-3.3974	0.995

[$] TaqMan Gene expression Assays identification number, * Amplicon length in base pairs, ^Coefficient of correlation, # Slope of Ct against 4-log dilution range [&] Amplification efficiency.

Supplementary materials and methods

Time course of the disease progression in mSOD1 mice. Please see the references listed at the end of the supplement. Some molecular markers of the disease in SOD1-G93A mice, including muscle mSOD1 aggregation, are observed as early as 30-40 days of age [1-4]. Loss of MN terminals commence at pre-symptomatic age of 40-60 days, those of fast muscles being first affected [5-9]. This is accompanied with decreased fast muscle contractile force and fiber cross-sectional area [1,10]. Clinical symptoms in SOD1-G93A mice, such as weakness, tremors and muscle wasting start to develop at about 90 days of age at which stage also slow muscle contractile force and ventral root neurons are affected [1,6,7,11,12]. At this stage, elevated levels of denervation marker Chrna1 has been described [13]. Hind-limb paralysis commences at about 120 days of age at which stage both fast and slow muscle fiber diameter is reduced and denervation is severe [1]. The SOD1-G93A become terminally paralysed at 4-5 months of age [11,12].

Calculations of relative gene expression. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method where the data are presented as a fold-change in gene expression normalized to the reference genes and relative to the non-transgenic age-matched calibrator. Briefly, the difference between the cycle threshold values (ΔCt) for the gene of interest (goi) and the geometric mean of the reference Ct-values for the same sample ($\Delta Ct = Ct_{goi} - Ct_{geom.\ mean\ of\ references}$) was calculated first for each mutant (n=5 per age) and age-matched wild type sample (n=5 per age). To obtain the difference (ΔΔCt) between the wild type (non-transgenic) calibrator and the mSOD1 samples, the mean of the ΔCt values of the wild type samples were subtracted from the mean ΔCt of the mSOD1 samples ($\Delta\Delta Ct$= Mean ΔCt_{mutant} - Mean ΔCt_{wt}). Because ΔCt is an exponential term, the linear difference in the expression was finally obtained by conversion to $2^{(-\Delta\Delta CT)}$. We designed the experiment to include calibrators and SOD1-G93A animals of one age in a single 96-well plate to exclude inter-run variations within each time point.

Western blot. PAX7 and MYOD displayed expected molecular weight of 60 kDa and 45 kDa, respectively. MYOG and MYF5 showed bands of approximately 32 and 35 kDa, respectively, when their expected sizes were 36 and 32 kDa.

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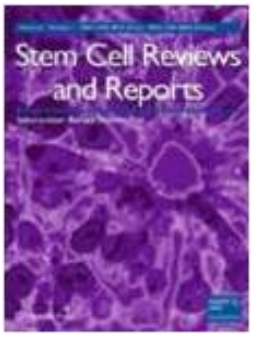
3.2. Stem Cell Reviews and Reports, 2011



Quantity and Activation of Myofiber-Associated Satellite Cells in a Mouse Model of Amyotrophic Lateral Sclerosis.

Manzano R, Toivonen JM, Calvo AC, Oliván S, Zaragoza P, Muñoz MJ, Montarras D, Osta R.

Stem Cell Rev. 2011 . DOI: 10.1007/s12015-011-9268-0

Stem Cell Rev and Rep
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Quantity and Activation of Myofiber-Associated Satellite Cells in a Mouse Model of Amyotrophic Lateral Sclerosis

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Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset motor neuron disease characterized by loss of motor neurons, progressive muscle atrophy, paralysis and ultimately, death. Although great majority of ALS cases are sporadic, 10% are inherited (familial ALS, fALS). Approximately 10%–15% of fALS cases are caused by mutations in *Cu/Zn superoxide dismutase* gene (*SOD1*) [1]. *SOD1* gene mutations described have permitted the generation of animal models that reproduce the main hallmarks of the human disease [2–4]. The most commonly used ALS model is an overexpresser of human *SOD1* carrying a mutation that substitutes a conserved glycine to alanine (SOD1-G93A) [5]. The SOD1-G93A animals reach symptomatic stage at approximately 90 days of age, showing signs of hindlimb weakness, impaired leg extension and shortened stride length. This proceeds to a complete paralysis of the limbs and to death by day 120–130 of age [6, 7] .

Although the most representative pathological consequence of ALS is the death of upper and lower motor neurons, there is good evidence indicating that the neuronal death is at least partially non-cell autonomous. Transgenic mice expressing mutant SOD1 (mSOD1) selectively in neurons [8, 9], astrocytes [10] or microglia [11, 12] did not lead to motor neuron degeneration. However, actuations directed to reduce mSOD1 in astrocytes or microglia [13, 14] or increase the percentage of wildtype cells in contact with cells carrying mSOD1 sharply delayed the disease progression and prolonged lifespan of the animal models [11, 15, 16]. Cells other than those of the CNS are also affected in ALS including fibroblasts [17, 18], lymphocytes [19] and, importantly, skeletal muscle [20, 21]. Muscle atrophy is among the earliest detectable pathologies in the SOD1-G93A animals [22], followed by alteration of the NMJs and retrograde axonal degeneration [23] and, finally, motor neuron death [24, 25]. This pattern of degeneration suggests that muscle abnormalities may precede motor neuron death rather than resulting from it. Skeletal muscle pathology and early functional abnormalities have been described in human sporadic and familial ALS [26–31] and in mSOD1 transgenic mouse models [32–34]. Recent studies suggest that skeletal muscle is one of the primary targets for mSOD1 mediated toxicity [35, 36]. Mice expressing mSOD1 under muscle specific promoters exhibit severe muscle disturbance including muscle atrophy, decreased functional performance, altered contractile and metabolic properties, sarcolemmal damage,

Electronic supplementary material The online version of this article (doi:10.1007/s12015-011-9268-0) contains supplementary material, which is available to authorized users.

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modulation of antioxidant enzyme activity and induction of reactive oxygen species (ROS). ROS accumulation in skeletal muscle also induced microglial activation and muscle atrophy [36, 37]. Distinct interventions in the skeletal muscle of mSOD1 mouse models, such as expression of growth factors and ablation of neurite growth inhibitor, result in ameliorated muscular phenotype, delay the disease progression, preserve neuromuscular junctions and prolong neuronal function and life expectancy [38–40]. Although the exact mechanisms that lead to the alleviation of the symptoms and pathology remain unknown, it has been suggested that one could be stimulation of skeletal muscle satellite cells (SMSCs) [38, 41, 42]. SMSCs are stem cells located at the periphery of the muscle fibers and are responsible for the protection and repair of muscle tissue during postnatal life. In adults, SMSCs normally remain quiescent. However, they may become activated, proliferate and differentiate upon acute injury, exercise or muscle denervation [43]. Paired-box transcription factor PAX7, one of the most widely accepted SMSC markers [44–48], is expressed in quiescent and activated satellite cells [49–51]. When activated, SMSCs re-enter the cell cycle, initiate the expression of a myogenic regulatory factor (MRF) *Myod1* (myogenic differentiation 1) and perform several rounds of proliferation. Subsequently, proliferating SMSCs downregulate *Myod1* and either elongate to form new myotubes that fuse each other or with the damaged myofiber to regenerate muscle or, alternatively, exit cell cycle as a mononuclear form to replenish quiescent satellite cell pool [43, 52, 53]. Because muscle is a primary target of mSOD1 toxicity and muscle-mediated therapies that ameliorate mSOD1 phenotype seem to act in part through the stimulation of SMSCs, it is of interest to characterize the SMSC number and activation status to investigate the role of those cells into ALS etiopathogeny. To this end, we have isolated individual skeletal myofibers from fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (SOL) muscles from SOD1-G93A and age-matched non-transgenic mice at different stages of the disease and determined the number of PAX7 and MYOD positive SMSCs per myofiber. We report a novel finding that both satellite cell number and activation status is modified at different stages of the disease in a mouse model of ALS, and these modifications vary depending on the muscle fiber composition.

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. The experimental mice were housed under a 12 h 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.

Myofiber Isolation

Postnatal (p) extensor digitorum longus (EDL) and soleus (SOL) muscles were harvested from male SOD1-G93A mice and their non-transgenic age-matched littermates at early presymptomatic (p40), late presymptomatic (p60), symptomatic (p90) and terminal stage (p120). Muscles were incubated in a DMEM+ GlutaMAX solution (Gibco) supplemented with 0.2% collagenase I (Sigma) for 1 h at 37°C. Detached individual myofibers were transferred to eppendorf tubes and immediately fixed in 10% paraformaldehyde (Sigma) for immunohistochemistry.

Immunohistochemistry

Myofibers were permeabilized with 0.5% Triton X-100 (Sigma) for 10 min at room temperature (RT), blocked with 20% goat serum (Sigma) for 30 min at RT and incubated with primary antibodies over night. Dilutions for the primary antibodies were 1:50 for mouse anti-PAX7 (DSHB) and 1:500 for rabbit anti-MYOD (Santa Cruz). PAX7/MYOD-stained myofibers were washed in 0.025% Tween-20 3 times and subsequently incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500) and Alexa Fluor 546 goat anti-rabbit IgG (1:3000) (Invitrogen) for 30 min at RT. Immunofluorescence was detected under a Nikon Eclipse 80i fluorescence microscope and pictures (Fig. 1) were captured using a Leica SP2 AOBS confocal scanning microscope. An average of 75 myofibers were counted for each age and muscle. SMSC number was determined as PAX7 positive cells in each myofiber and represented as the accumulative percentage of the total PAX7 positive cells, as described elsewhere [54]. Activation status at each age in SOD1-G93A and control groups was assessed from the number of PAX7+/MYOD+ and PAX7−/MYOD+ cells and expressed as percentage of the total number of cells counted [54].

Statistics

All statistis were performed using Statistica 5.0 (Statsoft). The influence of genotype (transgenic vs. control) and muscle type on PAX7 and MYOD expression was tested by parametric test two-way ANOVA followed by post hoc Tukey's HSD test for non-equal sample size when significant differences were found (the same approach was used in all comparisons). For all tests, p values less than 0.05 were considered significant.

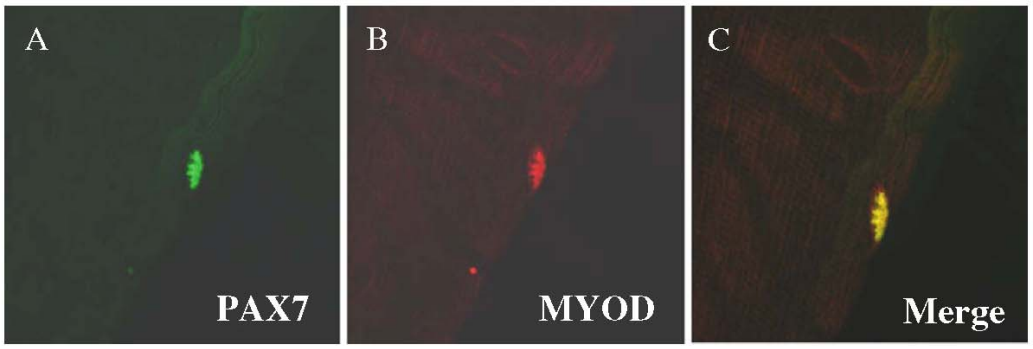

Fig. 1 Immunohistochemistry for PAX7 and MYOD in freshly isolated myofibers. Representative confocal microscope images showing the expression of **a** PAX7, **b** MYOD1, and **c** the coexpression (merge) of these proteins in freshly isolated myofibers

Results

Early Presymptomatic SOD1-G93A Mice Have Diminished Number of SMSCs

Satellite cell number in intact isolated myofibers derived from EDL and SOL muscles were quantified in SOD1-G93A and control mice based on their PAX7 expression, as previously described [46, 47, 54, 55]. The total numbers of animals used, myofibers counted and PAX7 positive cells found per myofiber are summarized in Table 1. The number of SMSCs in SOD1-G93A mice at p40 was significantly reduced compared to non-transgenic littermates (two-way ANOVA, $p=0.0002$ (EDL) and $p=0.0006$ (SOL)) and this decline was coincident in both EDL and SOL myofibers (Fig. 2a and b).

Muscle Type Strongly Influences SMSC Number in SOD1-G93A Mice

At late presymptomatic (p60) and symptomatic stage (p90) significant differences were found in SMSC numbers depending on the muscle type analyzed. At p60 EDL-derived muscle fibers possessed similar numbers of satellite cells in transgenic and control animals ($p=0.7507$) (Fig. 2c). In contrast, SOL muscles at p60 revealed a significant increase in the satellite cell pool suggesting an early induction of SMSCs in response to mSOD1 toxicity ($p=0.0057$) (Fig. 2d). As opposed to the previous stage, EDL muscle of SOD1-G93A at p90 presented an increased SMSC number per myofiber ($p=0.0028$) (Fig. 2e), whereas SOL transgenic myofibers showed a drastic diminution in satellite cell content ($p=0.0005$) (Fig. 2f). Finally, at p120, soleus derived SOD1-G93A myofibers depicted higher number of SMSCs ($p=0.0038$) (Fig. 2h). In the other hand, no differences were found in EDL myofibers between transgenic and control mice ($p=0.8440$) (Fig. 2g).

Notably, there was a significant interaction between muscle type and genotype variables at p60 and p90 which demonstrates that the muscle type influences how the SMSCs respond to mSOD1 expression and the effect of this influence is different depending on the stage of the disease (two-way ANOVA; p60 $F(1.269)=15.04$, $p<0.0001$; p90 $F(1.407)=38.22$, $p<0.0001$).

Overall, as previously described in wildtype mice [46, 53, 54, 56], SOL muscle contained higher number of SMSCs per myofiber than EDL in both transgenic and non-transgenic mice regardless of the age (two-way ANOVA, $F(1.115)=546.36$, $p<0.0001$) (Table 1).

Activation of SMSCs is Altered in SOD1-G93A Muscle Fibers

To get insight into the in situ activation status of SMSCs in myofibers, we co-stained EDL and SOL derived myofibers from SOD1-G93A transgenics and their control littermates at the above mentioned ages for PAX7 and MYOD. We then determined the percentage of myofiber-associated SMSCs that co-expressed these markers (PAX7+/MYOD+) as an indicator of activation status of these cells. SMSC expressing only MYOD (PAX7-/MYOD+) were also quantified. However, this group was not considered for statistical comparisons as it was found almost exclusively in p40 myofibers (Fig. 3a), showing no significant differences between transgenic and control groups (data not shown). We considered these cells as differentiating SMSCs involved in the normal growth of the muscle at this early postnatal stage.

At p40, we found no differences in MYOD+ cells between SOD1-G93A and control myofibers either in EDL or SOL, although a tendency towards a higher number of MYOD+ cells could be observed in SOD1-G93A mice (two-way ANOVA, $p>0.05$) (Fig. 3a). Following the tendency observed in the first stage, SOL SOD1-G93A myofibers at p60 revealed an increase in the number of MYOD+ SMSCs compared to the non-transgenic age matched myofibers ($p<0.0001$). On the other hand, at this

Table 1 Descriptive statistics of PAX7+ and MYOD+ cells per myofiber at different ages, phenotypes and muscles

	40 days				60 days				90 days				120 days			
PAX7	EDL n-Tg	EDL Tg	SOL n-Tg	SOL Tg	EDL n-Tg	EDL Tg	SOL n-Tg	SOL Tg	EDL n-Tg	EDL Tg	SOL n-Tg	SOL Tg	EDL n-Tg	EDL Tg	SOL n-Tg	SOL Tg
Minimum	4	2	1	2	1	1	8	9	1	2	5	1	2	3	3	1
Maximum	27	20	40	29	22	18	47	63	23	27	41	38	20	27	32	46
Median	12	8	22	18	10	8	19	27	7	10	21	15	7	8	15	19
Average	12,36	**8,03***	22,50	**15,88***	9,93	8,37	21,39	**27,71***	7,49	**10,73***	21,04	**15,79***	7,29	8,61	14,30	**18,46***
SD	5,21	3,59	9,90	7,69	4,15	3,70	9,45	11,48	3,79	4,82	7,72	10,37	3,03	4,45	6,35	11,48
SEM	0,57	0,45	2,02	1,57	0,39	0,58	1,00	2,17	0,29	0,52	0,74	1,62	0,23	0,77	0,50	1,53
MYOD	1	0,94	1	1,96	1	3,44	1	**8,53***	1	**6,84***	1	**8,17***	1	0,51	1	2,51
No.of mice	4	2	3	2	4	2	4	2	5	3	5	3	15	5	10	7
No.of myofibers	84	64	24	26	115	41	90	28	175	86	108	41	126	33	164	56

Descriptive statistics of the PAX7 and MYOD positive satellite cells in EDL and soleus muscle fibers from SOD1-G93A and control mice at different ages. In the table are detailed the PAX7 positive maximum, minimum, median, average, SD (standard deviation) and SEM (standard error of the mean). Also MYOD positive satellite percentage counts are expressed as fold change setting non-transgenic values for each group to 1. Significant differences by Post hoc Tukey HSD between the transgenic SOD1-G93A (Tg) and the control groups (n-Tg) for each age are showed in bold and marked with an asterisk. The number of mice used and the total number of fibers counted is shown for each group

stage, EDL SOD1-G93A myofibers showed a tendency towards increased MYOD+ cells although it did not reach significance (p=0.5504) (Fig. 3b). At symptomatic stage (p90), activated MYOD+ SMSCs were significantly more prominent both in EDL and SOL derived myofibers of SOD1-G93A mice (EDL, p=0.0548; SOL, p<0.0001) (Fig. 3c). Finally, at terminal stage (p120) the transgenic and control mice presented similar numbers of activated/proliferating MYOD+ SMSCs and this was consistent for both EDL and SOL derived myofibers (EDL, p=0.8862; SOL, p=0.5765) (Fig. 3d).

Discussion

In the SOD1-G93A model of ALS, the earliest pathological signs have been described in the skeletal muscle at around 40 days of age with aggregation of mSOD1 proteins [57], loss of tetanic contraction force and a decreased number of functional motor units [58]. Overt symptoms appear approximately at 90 days of age with fine tremors and weakness of the hindlimbs [6, 7] and proceed with the loss of motor neurons toward the later stages where a reduction in the central neuronal cell bodies becomes evident [58]. The muscle pathology occurs earlier and is more pronounced in slow-twitch compared to the fast-twitch muscle [23, 32, 58–60]. However, the status of SMSCs responsible for regenerative capacity of the muscle has not been previously studied. Here, we investigated SMSC number and activation in situ in freshly isolated myofibers derived from groups of SOD1-G93A and control animals of age 40–120 days. This enabled us to follow the time course of satellite cell alterations from phenotypically unaffected stage to the severely semi-paralytic terminal stage. Because muscle type susceptibility to mSOD1 toxicity varies, we investigated SMSC quantity and activation using typical fast twitch EDL, composed approximately half and half of fast glycolytic (type IIb) and fast oxidative-glycolytic (type IIa) fibers, as well as in typical slow twitch SOL containing approximately 60% slow oxidative (type I) fibers and 40% type IIa fibers [61]. In the control animals (see Table 1), the number of PAX7+ SMSCs was similar to previous reports that used PAX7 immunodetection or in nestin-GFP reporter [46, 54, 62, 63]. Slow myofibers showed a wider range (max-min) of SMSCs per myofiber, as seen from relatively linear cumulative percentage curves for SOL compared to the typically sigmoidal curves of the EDL fibers, and contained more SMSCs that fast fibers, also reported earlier [54, 62]. This suggests that the methodology used is robustly repeatable between different laboratories.

At early presymptomatic stage (p40), both fast and slow muscles presented a shift toward a lower number of PAX7+ SMSCs per myofiber. Because the motor unit numbers in

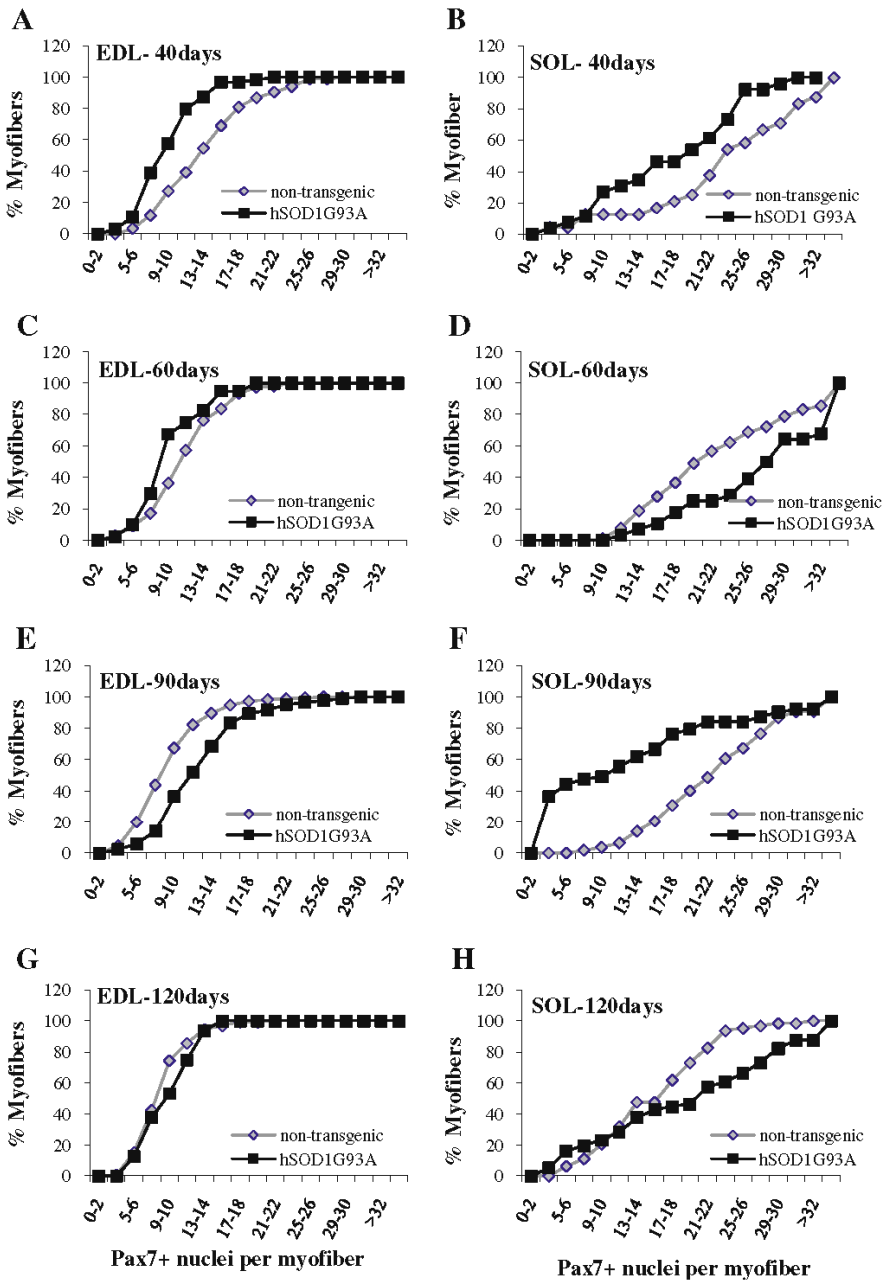

Fig. 2 Cumulative percentage of PAX7+ cells in isolated myofibers from SOD1-G93A and control mice at age of p40, p60, p90 and p120. Quantification of PAX7 immunolabelled satellite cells in freshly isolated EDL and SOL derived myofibers from SOD1-G93A (black squares) and non-transgenic control mice (*grey diamonds*). **a** EDL and **b** SOL fibers at p40, **c** EDL and **d** SOL fibers at p60, **e** EDL and **f** SOL fibers at p90, **g** EDL and **h** SOL fibers at p120. Data points indicate the cumulative percentage of myofibers (out of total myofibers counted, y-axis) that contain indicated number of satellite cells (X-axis). Therefore, the shift of the curve to the right represents higher content of PAX7 immunolabelled satellite cells per fiber

the presymptomatic mSOD1 animals are affected in fast EDL but not in slow SOL muscle [58, 59], it is unlikely that the early alterations in SMSC content are directly related to the loss of nerve to muscle relationship. The lack of significant satellite cell activation measured as MYOD+ cells also suggests that the satellite cell pool in presymptomatic mSOD1 muscles is not exhausted as a result of increased SMSC activation and differentiation. Therefore, it is possible that diminished number of PAX7+ cells reflects a direct consequence of the mSOD1 expression on satellite cell quantity, possibly related to oxidative stress that have been reported in ALS patient derived myoblasts [25, 64]. As opposed to p40, where only non-significant tendency towards SMSC activation was observed, an 8.5-fold relative increase in activated (PAX7+/MYOD+) SMSCs occurred in SOD1-G93A SOL myofibers at late presymptomatic stage (p60). Consistently, the number of PAX7+ cells was also increased in the SOL derived myofibers at

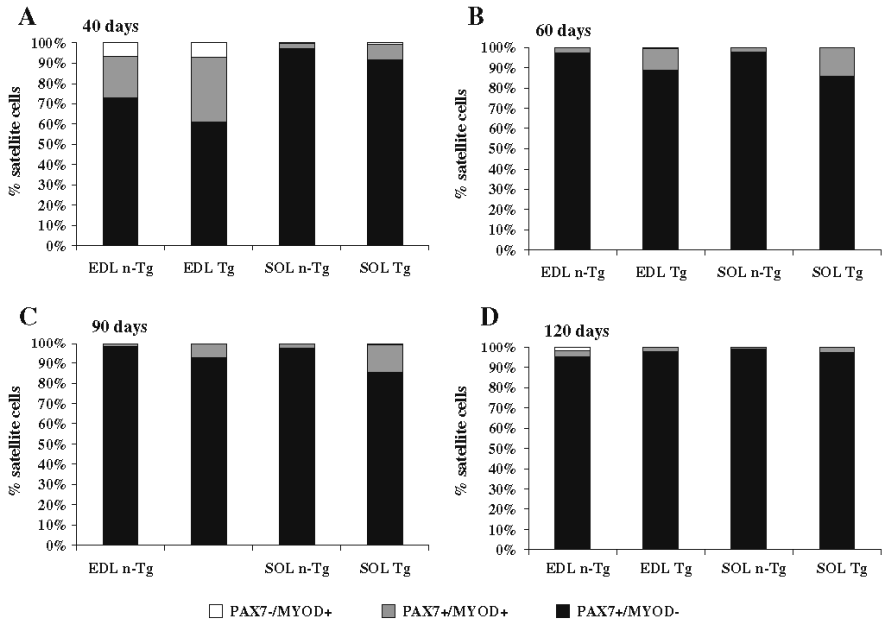

Fig. 3 Percentage of quiescent and activated/proliferating satellite cells in isolated myofibers. PAX7 and MYOD double-immunolabelling was carried out in freshly isolated myofibers from EDL and SOL muscles of SOD1-G93A (Tg) and non-transgenic (n-Tg) control mice at **a** p40, **b** p60, **c** p90 and **d** p120. Different segments of the stack bars represent the percentage of cells that express indicated marker(s): PAX7+/MYOD− (*black*); PAX7+/MYOD+ (*grey*) or PAX7−/MYOD+ (*white*)

this age. This may reflect a response to replenish the reduced pool of SMSCs observed at earlier and/or to repair the commencing muscle damage described [65]. In contrast, EDL myofibers presented only non-significant tendency to activate the satellite cell pool and relative shift towards more wild type-like quantity of PAX7+ cells. At symptomatic stage (p90) where slow muscle denervation commences [23, 58, 59], SOL myofibers from mSOD1 animals maintained relatively higher capacity to become activated. Curiously, the number of PAX7+ cells was diminished suggesting that the enhanced activation may cause transient depletion of SMSC pool. On the other hand EDL myofibers, which in SOD1-G93A mice are seriously denervated at this age, experienced significant (6.84-fold) activation at p90 which correlated with the pronounced increment in the quantity of PAX7+ cell pool. This possibly reflects the combined regenerative attempt in response to the mSOD1 muscle toxicity and denervation. Finally, at terminal stage of p120, no signs of altered SMSC activation were present in SOD1-G93A myofibers. However, significant increase in PAX7+ cell population was observed in SOL myofibers. Once again, the lack of activation (increased quiescence) in the SOL at p120 may contribute to the more normal levels of SMSCs in the slow muscle at this stage. Alternatively, the effect of denervation may cause late stimulation of PAX7+ SMSCs in the terminal stage SOL muscles. The differential response of fast and slow muscles was statistically confirmed as a significant interaction between muscle type and genotype variables.

Although the early diminution in the amount of PAX7+ cells in SOD1-G93A muscles is clear, the interpretation of the observed changes in SMSC dynamics along the later stages of the disease is less straightforward. Several contributing factors, including satellite cell-autonomous toxicity of mSOD1 as well as those potentially brought upon by reduced neural stimulation or altered paracrine signalling at the natural stem cell niche are possible. mSOD1 is expressed in equal levels in slow and fast muscles based on mRNA expression (Supplementary Figure 1) and protein data [35]. Both SOL and EDL derived mSOD1 satellite cells are also capable of differentiation into myotubes in vitro (Supplementary Figure 2 and Supplementary Video 1). However, we cannot exclude the possibility that subtle mSOD1-dependent differences in the myogenic process between the two muscles exist, and elucidation of these alterations warrants further studies.

As already mentioned, mSOD1 mice exhibit characteristic distal axonopathy where neuromuscular connections are first lost from the fast muscles (such as EDL) connected with more vulnerable large motor neurons. In slow muscles (such as SOL) the denervation commences only at the symptomatic stage [23, 58, 59]. Short-term surgical denervation, such as sciatic axotomy, stimulates myofiber-attached SMSCs activation in the affected muscles [66], whereas long term denervation leads to exhaustion of SMSC pool with yet undetermined mechanism. To our knowledge, direct contacts with SMSCs and motor nerve endings have not been reported. The effects of decreased electrical activity on SMSCs are therefore likely to occur indirectly through signals from the affected muscle. Thus, alterations in gene expression on whole mSOD1 muscle tissue may give insight to processes that influence SMSC

balance in the myofibers. Both surgical denervation and SOD1-G93A expression transcriptionally upregulate myogenic programme in vivo in whole muscles [65]. *Pax7* mRNA was found to be relatively upregulated in presymtomatic SOD1-G93A mice (at p60), albeit significant protein increment by western blot was detected only at p90. Interestingly, increased *Pax7* expression at p60 was coincident with dramatic upregulation of *Rrad*, a marker sensitive to oxidative stress, and preceded that of denervation marker *Chrna1* (acetylcholine receptor alpha subunit). The relative contribution to these gene expression changes of transcripts derived from SMSCs and mature myofibers in unknown, except for *Pax7* which is satellite cell-specific. However, the implication from the marker gene expression is that skeletal muscle oxidative stress is probably an early consequence of mSOD1 expression. Although loss of motor units in the fast muscles commences early, denervation only becomes sufficiently severe to induce *Chrna1* expression at 75 to 90 days of age [65, 67]. Therefore, we propose that the decreased SMSC reserve observed at p40 is stimulated at p60 by yet uncharacterized signals caused by commencing muscle damage. Whether already described molecular signals, such as increased ROS production, contribute to SMSC induction in mSOD1 animals remains to be elucidated.

As the outcome in ALS and its models is severe muscle atrophy, it is clear that the attempt to overcome the muscle damage and denervation by SMSC upregulation or stimulated myogenic programme is ultimately in vain. This potentially bears significance to the potential therapeutic use of myogenic precursors to treat the disease. It will be important to determine if the SMSCs in the fALS patients and the mSOD1 animal models are functionally impaired, if the abortive attempt at myogenesis ultimately a result of metabolic alterations in the mature myofibers, or both. Recent study suggests post-transcriptional alterations caused by mSOD1 expression may result in blunted regenerative response in SOD1-G93A muscle [65]. Therefore, further studies addressing potential downstream processes disturbed in the ALS muscles and myogenic precursors are in demand. Positive effect on satellite cell number and activation status upon treatments with IGF-1 or its variants in mSOD1 mice [38] and in ALS patient-derived myoblasts [41] presents encouraging prospects for development of therapeutic interventions. Furthermore, the potential role of chemokine-mediated recruitment of myogenic precursors from extramuscular tissues deserves a further look, as two chemokines and chemokine receptor *Cxcr4* have been recently shown to be upregulated in SOD1-G93A muscle [65].

To conclude, our data is consistent with the view that SMSCs in the SOD1-G93A muscle are affected even prior to loss of motor units or decrease in muscle contraction force. mSOD1 induced alterations in the quantity of myofiber-associated SMSC varies depending on muscle type, slow myofibers being more rapidly activated at the onset of muscle damage. As metabolic changes in the skeletal muscle can promote denervation [25, 36], it remains to be seen if the more rapid activation SMSCs at early presymptomatic stage may partially explain the relative resistance of slow muscles to the motor unit loss in mSOD1 mice.

Acknowledgements The authors thank María Royo and Mamen Carreras (Microscopy and Image Service) for excellent technical assistance and help with confocal microscope, and the I+ CS/IIS Aragon (Instituto Aragonés de Ciencias de la Salud) for access to the microscope and Barbara Gayraud Morel from the Department of Developmental Biology of the Institut Pasteur for her technical and methodological assistance . This study was supported by the grant of CAJA NAVARRA: "Tú eliges, tu decides"; PI071133 and PI10/01787 from the Fondo de Investigación Sanitaria of Spain and PAMER from the Instituto Aragonés de Ciencias de la Salud (PIPAMER 09/09).

Conflicts of interest The authors declare they have no conflict of interest.

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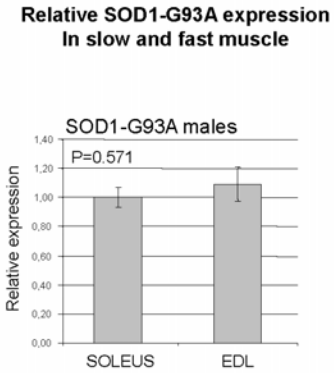
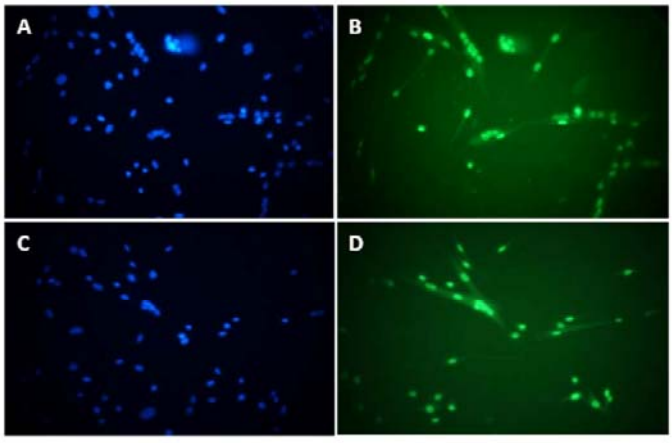


Supplementary Figure 1 hSOD1-G93A is equally expressed in cultured primary myoblasts derived from soleus (slow) and EDL (fast) muscles. The data shows mean (+/- standard error of mean) expression of human SOD1 from four replicate satellite cell cultures of hSOD1-G93A transgenics. No hSOD1 expression was observed in satellite cell cultures derived from wild-type animals.



Supplementary Figure 2 Satellite cell cultures from EDL and soleus muscles of SOD1G93A transgenic mice. Myogenin expression was analysed by immunocytochemistry after induction of differentiation 4.5 days post plating. Nuclear Hoechst staining from **A** EDL and **C** SOL. Myogenin staining from **B** EDL and **D** SOL.

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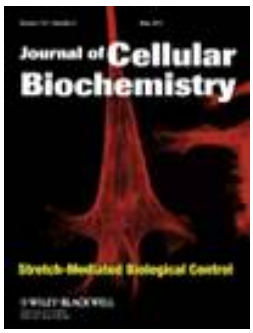

Sex, Fiber-Type And Age Dependent In Vitro Proliferation Of Mouse Muscle Satellite Cells.

Manzano R, Toivonen JM, Calvo AC, Miana-Mena FJ, Zaragoza P, Muñoz MJ, Montarras D, Osta R.

J Cell Biochem. 2011. DOI: 10.1002/jcb.23197

ARTICLE
Journal of Cellular Biochemistry 9999:1–13 (2011)
JCB-11-0251(23197)
Journal of Cellular Biochemistry

Sex,[Q1] Fiber-Type, and Age Dependent In Vitro Proliferation Of Mouse Muscle Satellite Cells

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ABSTRACT

During postnatal growth and after muscle injury, satellite cells proliferate and differentiate into myotubes to form and repair musculature. Comparison of studies on satellite cell proliferation and differentiation characteristics is confounded by the heterogeneity of the experimental conditions used. To examine the influence of sex, age, and fiber-type origin on in vitro properties of satellite cells derived from postnatal muscles, fast extensor digitorum longus (EDL) and slow soleus (SOL) muscles were extracted from male and female mice of 1 week to 3 months of age. Myoblast proliferation and myogenic regulatory factor (MRF) expression was measured from cultures of freshly isolated satellite cells. Higher proliferation rate and elevated *Myod1* expression was found in male EDL and SOL derived cells compared with females at age of 40, 60, and 120 days, whereas inverse tendency for cell proliferation was apparent in EDL of juvenile (7-day-old) pups. *Myogenin* and *Mrf4* transcripts were generally elevated in males of 40 and 60 days of age and in female EDL of juveniles. However, these differentiation markers did not significantly correlate with proliferation rate at all ages. *Pax7*, whose overexpression can block myogenesis, was up-regulated especially in 40-day-old females where MRF expression was low. These results indicate that gender, postnatal age, and muscle fiber origin affect proliferation and muscle transcription factor expression in vitro. The results also support the view that satellite cells originating from slow and fast muscles are intrinsically different and warrant further studies on the effect of cell origin for therapeutic approaches. J. Cell. Biochem. 9999: 1–13, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: MUSCLE; SATELLITE CELL; PROLIFERATION; AGE; SEX; MUSCLE FIBER; CELL CULTURE

Sexual dimorphism affects various aspects of the human and animal life. Body size, life expectancy, and susceptibility to diseases differ in males and females (Deasy et al., 2008). Skeletal muscle among other tissues presents unequal characteristics in the two sexes. Muscle fiber diameter and, therefore, muscle mass is *generally* higher in males, although no differences are found in terms of maximum force generated when normalized by muscle mass (Miller et al., 1993; Kanehisa et al., 1996). On the other hand, female muscles posses consistently greater fatigue resistance than male muscles under submaximal contractions (Hicks et al., 2001). Purely structural, mechanistic, or metabolic differences in the musculature do not sufficiently explain the underlying mechanisms for muscle mass differences in different sexes which, partly because of lack of animal model studies, still remain unclear.

Muscle mass is related to the fiber cross-sectional area, which is dependent on muscle stem cell activity as these cells are responsible for postnatal muscle growth and repair (Deasy et al., 2008). The best documented muscle stem cells are satellite cells, committed mononuclear progenitors derived from the embryonic dermomyotome that reside between the basal lamina and the sarcolemma of the mature muscle fiber. Several molecular markers have been used to identify satellite cell population, paired-box transcription factor *Pax7* being the most widely accepted (Seale et al., 2000; Zammit et al., 2004; Shefer et al., 2006; Buckingham, 2007). In adults, satellite cells are quiescent but become activated upon stimuli such as acute muscle injury, exercise, or denervation (Seale and Rudnicki, 2000). The activation is associated with coordinated expression of myogenic regulatory factors (MRFs) (Smith et al., 1994; Cornelison

Grant sponsor: CAJA NAVARRA; Fondo de Investigación Sanitaria of Spain and Instituto Aragonés de Ciencias de la Salud; Grant numbers: PI071133, PIPAMER 09/09.
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Received 11 May 2011; Accepted 12 May 2011 • DOI 10.1002/jcb.23197 • © 2011 Wiley-Liss, Inc.
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and Wold, 1997; Cooper et al., 1999), muscle-specific basic helix-loop-helix (bHLH) transcription factors that heterodimerize with ubiquitously expressed bHLH transcription factors named E proteins to drive the expression of muscle specific genes. Upon activation satellite cells re-enter the cell cycle, downregulate *Pax7* (Olguin and Olwin, 2004; Zammit et al., 2004), upregulate the primary MRF *Myod1*, and perform several rounds of proliferation before upregulating a secondary MRF *myogenin* (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994), and subsequently Mrf4 [Seale and Rudnicki, 2000). In the end of this coordinated process, the proliferating satellite cells (also called myoblasts) begin to fuse with each other and differentiate into myotubes or, alternatively, fuse with existing muscle fibers to repair damage or to increase muscle mass (Hawke and Garry, 2001; Charge and Rudnicki, 2004).

Because of their central role in regulation of muscle-specific genes, it is possible that *Myod1* and *myogenin* expression may have direct effects on muscle bulk. In this scenario, increased muscle mass in males would be expected to be associated with higher expression of the MRF genes compared with females. However, comparison of MRF expression in rat anterior tibial muscle revealed no gender differences *Myod1* gene expression and even higher *myogenin* mRNA levels in females than in males (te Pas et al., 1999), suggesting that gender-related differences in the intact muscle cannot be primarily explained by MRF expression levels. Muscle cell lines derived from turkeys selected for increased muscle mass and from related but randomly bred control animals revealed higher in vitro proliferation and differentiation rates in the selection line, whereas there were no sex-dependent differences within the selected or control groups (Velleman et al., 2000). Consistently, subsequent in vitro MRF expression analysis revealed no drastic sex-dependent differences in *Myod1* or *myogenin* expression, although there was an increment in the *Myod1* expression in highly proliferative (selected) cell line compared with the slower proliferating control cell line (Liu et al., 2005). The authors suggest that this in vitro result may indicate prolonged proliferation of the satellite cells also in vivo which would, in turn, promote the formation of large myotubes with higher number of nuclei and ultimately result in increased muscle mass.

With respect to satellite cell content ex vivo, no sex differences have been reported in mouse gastrocnemius (Salimena et al., 2004) or in human vastus lateralis (Roth et al., 2000) and tibialis anterior muscle (Kadi et al., 2004), although age-dependent reduction has been reported in one *study* (Kadi et al., 2004). Discrepancies also exist in reported differentiation capacity of satellite cells in cell culture. Higher differentiation rate of male myoblasts with respect to females was reported in turkey pectoralis major-derived cells, although the proliferation rate was similar in both sexes (Velleman et al., 2000). In another study using the same muscle and age range (Doumit et al., 1990), no differences in proliferation or differentiation was found. Gender-dependent heterogeneity may also affect the regenerative efficiency of satellite cells when transplanted in muscle-compromized hosts. Although muscle derived stem cells from both genders have been used to characterize their in vivo regenerative capacity (Lee et al., 2000) direct comparisons of male and female derived stem cells have not been carried out.

Besides the sex of the donor, muscle fiber type has been reported to influence satellite cell properties (Rosenblatt et al., 1996; Huang et al., 2006; Rossi et al., 2010). Two main types of muscle fibers exist based on their expression of myosin heavy chain isozymes. Type I fibers (slow-twitch fibers) exhibit slow myosin ATPase activity and oxidative metabolism, present high fatigue resistance and are implicated in postural maintenance and endurance. By contrast, type II fibers (fast-twitch fibers) express fast myosin ATPase activity and anaerobic (glycolytic) metabolism, present low fatigue resistance, and participate in fast corporal movements. Although most muscles are composed of a mixture of both fast and slow fiber types, the percentage is highly variable among the different muscles of the body. Fibers in slow-twitch muscle contain more satellite cells than those in fast-twitch muscles, as they are the first and most frequently recruited during muscle activity (Hawke and Garry, 2001; Collins et al., 2005[Q2]). However, the satellite cell content in the muscles composed of a mixture of slow and fast-twitch fibers show no fiber type-specific differences in humans (Kadi et al., 2006). Throughout life, the composition of myofibers is not stable but may experience dynamic changes, for example, in response to exercise, injury, aging, or disease (Canepari et al., 2010).

The two most commonly used "prototypic" fast and slow muscles are extensor digitorum longus (EDL) and soleus (SOL), respectively. Comparison of rat EDL and SOL muscles at age of 1 month, 1 year, and two years revealed a continuous decrease in satellite cell number with age in EDL but an increase up till 1 year of age in SOL (Gibson and Schultz, 1983). Not only the number of satellite cells is different in fast and slow-twitch fibers, but also their potential for proliferation and differentiation in vitro may differ. Studies of male rats 2 months of age indicated that SOL-derived satellite cells displayed a higher proliferative potential but lower differentiation rate compared to those derived from EDL (Lagord et al., 1998). Moreover, the capacity of EDL and SOL satellite cells to regenerate fast (type II) and slow (type I) muscle, respectively, is not reversed by electrical stimulation resembling firing patterns of the opposite muscle type (Kalhovde et al., 2005). This suggests that fast and slow skeletal muscle fiber associated satellite cells are intrinsically different. Differences in MRF expression in fast- and slow-twitch muscle fibers have been also described. *Myod1* is preferentially expressed in the fast EDL whereas *myogenin* is mainly distributed in the slow soleus muscle (Voytik et al., 1993). However, the biological significance of this finding remains obscure.

The analyses of satellite cell numbers in young and aged humans (Renault et al., 2002), pigs (Campion et al., 1981), and rodents (Gibson and Schultz, 1983; Shefer et al., 2006) indicated a decline in satellite cell number with age, although in some cases muscle type-dependent increases in early life were found. I young mice, decrease in the number of EDL-associated satellite cells starts earlier that in SOL (Gibson and Schultz, 1983). Comparison of EDL and SOL muscles from young and aging mice revealed that, in EDL, a major decline in satellite cell number occurs before 1 year of age, whereas in SOL this only occurs by the age of 2.5 years (Shefer et al., 2006), consistently with accelerated aging-related atrophy of fast muscles. The proliferation and differentiation rate of satellite cells in vitro has been also shown to decrease as function of age in pigs (Mesires and Doumit, 2002). In turkeys, however, no age-associated differences in cell proliferation were found (Doumit et al., 1990). In male mice, initial proliferative phase in vitro is retarded in satellite cells derived

from senile animals (29–33 months old) compared with young males (3–6 months), although differentiation capacity is not affected (Shefer et al., 2006).

Newborn or very young animals are commonly used as a source of muscle stem cells because they are present in higher numbers compared with adult muscle (Hawke and Garry, 2001). However, little is known about potential differences in proliferation dynamics in newborn, pre-puberal, puberal, and young adult individuals. Notably, studies that investigate these parameters in parallel using both sexes and different muscle types do not exist. This bias may derive from the fact that at this stage, the musculature is in the growing phase and show altering satellite cell dynamics in vivo (White et al., 2010). Additionally, changes in male and female sex hormones in the young animals may influence satellite cell function (Chen et al., 2005; Enns and Tiidus, 2008). All evidence presented imply that the age and sex of the individual, as well as muscle fiber-type can strongly influence satellite cell physiology and may provide an explanation for some of the contrasting data obtained from seemingly similar experiments. Importantly, biased use of only one sex (normally males) in the studies of the muscle stem cell function or their therapeutic use should be discouraged (Check Hayden, 2010). Hence, further research is necessary to clarify the number, proliferation, differentiation, and MRF expression of satellite cells as these conclusions may condition research and therapeutic approaches. The aim of this study was to investigate the in vitro proliferation rate and MRF expression of the mouse satellite cells during rapid postnatal growth and muscle maturation. Satellite cells extracted from fast and slow-twitch skeletal muscles of male and female mice were studied in parallel.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethics Committee of Universidad de Zaragoza and followed the international and the institutional guidelines for the use of laboratory animals. Mice were housed under a 12 h light: 12 h dark cycle in 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 and female B6SJL mice per age group were sacrificed at 7 (neonatal), 40 (early-young), 60 (young adults), and 120 days of age (adults). The EDL and the SOL muscles from both sexes were collected and processed in parallel. Satellite cell extraction and culture was carried out as described (Montarras et al., 2005). Briefly, pools of six muscles per age, sex, and muscle type were minced to a slurry with sterile dissection scissors and digested in F-12 + GlutaMAX nutrient mixture (Gibco) containing 0.1% trypsin and 0.1% collagenase (w/v) (Sigma–Aldrich) at 37°C for 30 min. The supernatant was collected and further enzymatic digestion was inhibited by addition of fetal bovine serum (Gibco). Three to four rounds of digestion were performed until the muscle bulk was digested completely. Pooled cell suspension from each group was filtered through a 70 μm diameter sterile strainer and centrifuged 1,800 rpm for 15 min at 4°C. Cell pellet was resuspended to DMEM + GlutaMAX (Gibco) and stained with 0.4% trypan blue (Sigma–Aldrich) for viable cell counting. For each well used for proliferation and gene expression analysis (see below), total 1,000 cells were seeded in 96-well plates covered with 0.1 mg/ml of Matrigel basement membrane matrix (Becton Dickinson SA). The culture medium consisted of 39% F-12 + GlutaMAX (Gibco), 39% DMEM + GlutaMAX (Gibco), 10% fetal calf serum (Gibco) and 2% Ultroser G (Pall-Biosepra). This medium was used as it supports both proliferation and differentiation of satellite cells without requirement for switch to differentiation medium. Cells were left to adhere and start proliferating for 3.5 days at 37°C and 5% CO_2. At least four replicate wells for proliferation assay and for PCR analysis were plated for each group of sex, muscle type, and age. The experiments were performed in parallel with the cell proliferation and the gene expression analysis.

CELL PROLIFERATION ASSAY

Starting 3.5 days after the plating, and repeatedly every 24 h till 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 (Sigma–Aldrich) for 5 min. After washing with 1× PBS, five fields per well were photographed at 20× magnification under an epi-fluorescence microscope (Nikon TE2000-E) at 325 nm. For each time point, the total number of cells from five random fields per replicate (total 20 fields) was counted from each sex, muscle type, and age. At each day of the culture, the data were compared with the corresponding sex and age group of interest (see below for the statistics). These data were also normalized so that the maximum value from each comparison is set to 100. The error bars represent the standard error as percentage of the maximum value of the comparison that has been set to 100. The *P* values for each data point are shown in Supplementary online materials (Table 1).

QUANTIFICATION OF MRF EXPRESSION

In parallel with cell proliferation plates, plates for RNA extraction were washed with cold 1× PBS and transferred directly on 96-well plates to −80°C to wait extraction. RNA was extracted and the cDNA was synthesized using the Cells-to-cDNA kit (Ambion) according to manufacturer's instructions. Plates were placed on ice and cells were lysed in 100 μl of ice cold cell lysis solution, followed by heat treatment at 75°C for 15 min to rupture cells and to eliminate endogenous RNase activity. 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 from each RNA sample was performed in duplicates. Briefly, 2 μl of dNTPs and 1 μl of random hexamers were mixed with 5 μl RNA followed by incubation at 75°C for 3 min. Two microliters of master mix composed of 1 μl 10× reverse transcription buffer, 0.5 μl M-MLV retrotranscriptase, and 0.5 μl RNase inhibitor were added and the reaction was incubated at 42°C for 60 min followed by 95°C for 10 min. For QPCR, cDNA was diluted 1:10 in dH_2O and 2 μl were used as a template for each reaction (three replicates per cDNA sample) containing 2.5 μl Fast 2X TaqMan master mix (No AmpErase UNG) and 0.5 μl gene-specific TaqMan assays (Applied Biosystems) for *Pax7* (Mm00834079_m1), *Myod1* (mM00440387_m1), *myogenin*

(Mm00446194_m1), or Mrf4 (Myf6, Mm00435126_m1) in a final volume of 5 µl. Reactions were run using StepOne Plus Real-Time PCR System (Applied Biosystems) using following cycle: 95°C for 10 min, followed by 47 cycles of 95°C for 15 s and 60°C for 30 s. Reaction efficiencies of the primer/probe sets were inside 100 ± 10% over 4-log dilution range of template RNA prepared in the above mentioned method. In the same conditions, reference gene amplification was performed using TaqMan assays for three reference genes (Applied Biosystems): 18S ribosomal RNA (4352930E), Gapdh (4352932E), and β-actin (4352933E). Geometric mean of these housekeeping genes was used for normalization and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method (Manzano et al., 2011 and references therein). Ct values for the housekeeping genes and the calculated geometric mean are shown in Supplementary online material (Fig. 1). Because Ct value is exponentially related to copy number, the upper and lower error values for relative expression were estimated from mean change in Ct (ΔΔCt) plus standard deviation (ΔΔCt + SD) amd ΔΔCt minus standard deviation (ΔΔCt − SD) for each group (Figs. 6 and 7, Supplementary online material Table 2).

STATISTICAL ANALYSIS

Total cells from five photographic fields of a single well were counted and mean and standard error of mean (SEM) for each biological sample was calculated from the value of four replicate wells. Different groups were compared using Student's *t*-test (Statistic 5.0). Statistical differences were considered significant at $P < 0.05$.

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In order to ensure the myogenic origin of the studied cells, one well per group was fixed at day 4.5 of cell culture and immunostained for MYOD1, a marker for activated (proliferating) myoblasts. Cells were rinsed with PBS and fixed in 4% neutral buffered formalin solution (Sigma–Aldrich). Fixed cells were permeabilized with 0.2% (v/v) Triton X-100 (Sigma–Aldrich) in PBS and blocked using 0.2% (w/v) gelatine in PBS. Monoclonal antibody for MYOD1 (sc-304, Santa Cruz Biotechnology) was diluted 1:100 in *PBS* and the fixed/permeabilized cells were incubated with the primary antibody for 2 h at room temperature. The primary antibody was visualized with Alexa 546-conjugated anti-rabbit secondary antibody (Invitrogen) before mounting in DakoCytomation Faramount fluorescent mounting medium containing 1,000 ng/ml Hoechst 33342 (Sigma–Aldrich). All cultures used for the study showed myogenic (MYOD1 positive) cell content 70–80%.

RESULTS

To investigate potential sex- and muscle type-dependent variation of in vitro proliferation rate of satellite cells from young and mature wild-type mice, satellite cells were extracted from typical fast-twitch EDL and slow-twitch SOL muscles from both sexes at age of 7, 40, 60, and 120 days (see Materials and Methods Section for details). From all muscle, gender, and age groups, the cells were seeded at standard density immediately after the extraction to avoid potential effects from ex vivo amplification and cryopreservation. General proliferation rate was measured from the total number of Hoechst-stained nuclei per well every 24 h between 3.5 days (no differentiation evident) and 7.5 days (differentiation to myotubes evident) of culture (Fig. 1A). Immunostaining of fixed cells at day 4.5 revealed that 70–80% of the cells were expressing MYOD, a marker for activated myoblasts (Fig. 1B). In conditions used for these studies, differentiation into myotubes was observed in both sexes and all ages and muscle types.

In satellite cells (hereafter called myoblasts) derived from the muscles of 7-day-old mice, significantly faster proliferation was observed EDL-derived myoblast of females (Fig. 2A), whereas in SOL there were no consistent differences between sexes at any time point (Fig. 2B) (see Supplementary Table 1 for *P* values for each data point). However, fast-twitch EDL-derived cells proliferated consistently faster than slow-twitch SOL-derived cells, independently of gender (Fig. 2C and D), although in males the substantial sample variation precluded significance in last time points. In contrast, in myoblasts from 40-day-old animals a tendency for higher proliferation rate was observed in males compared to females and in both muscles under study (Fig. 3A and B). However, this difference was not significant at every day of culture due to sample variation between the four replicates. As in earlier age, EDL-derived cells tended to proliferate faster than those from the SOL, although this was only significant in females (Fig. 3C and D). At 60 days of age, myoblast proliferation was again higher in males compared with females in both muscles (Fig. 4A and B). Proliferation rate of EDL-derived myoblasts continued to be higher compared with that of SOL-derived cells in both sexes (Fig. 4C and D). Finally, like at 60 days of age, myoblasts from animals of 120 days of age proliferated faster in males than in females in both in EDL and SOL-derived cultures (Fig. 5A and B). However, unlike in younger animals, muscle type comparison from same gender revealed that EDL and SOL-derived myoblasts proliferate with similar rate and suggested possibly even higher proliferation rate in SOL-derived cells in females (Fig. 5C and D). Collective data from these primary myoblast proliferation experiments indicate that in early juveniles (7 days), male satellite cell derived myoblasts show equal or lower proliferation capacity compared with females. Later, however, their proliferation rate exceeds that of female-derived cultures. Cells from fast EDL muscle, on the other hand, are generally more proliferative that those of slow SOL muscle in vitro when derived from young animals up to at least 2 months of age. However, this difference is abolished (males) or possibly reversed (females) in more mature mice of 120 days.

Because our findings with respect to proliferation rate contradicts those previously reported in 60-day-old rats where SOL was found to proliferate faster than EDL (Lagord et al., 1998), we looked for further evidence for our findings on molecular level. Satellite cell proliferation that precedes the differentiation into myotubes in vitro and in vivo is characterized by up-regulation of *Myod1* mRNA (Megeney et al., 1996; Zammit et al., 2004). Therefore, higher *Myod1* mRNA levels would be expected in myoblasts that proliferate faster. To investigate if the observed gender-specific modulation of myoblast proliferation may be reflected in the level of MRFs we analyzed *Myod1* expression at two time points of cell culture: At day

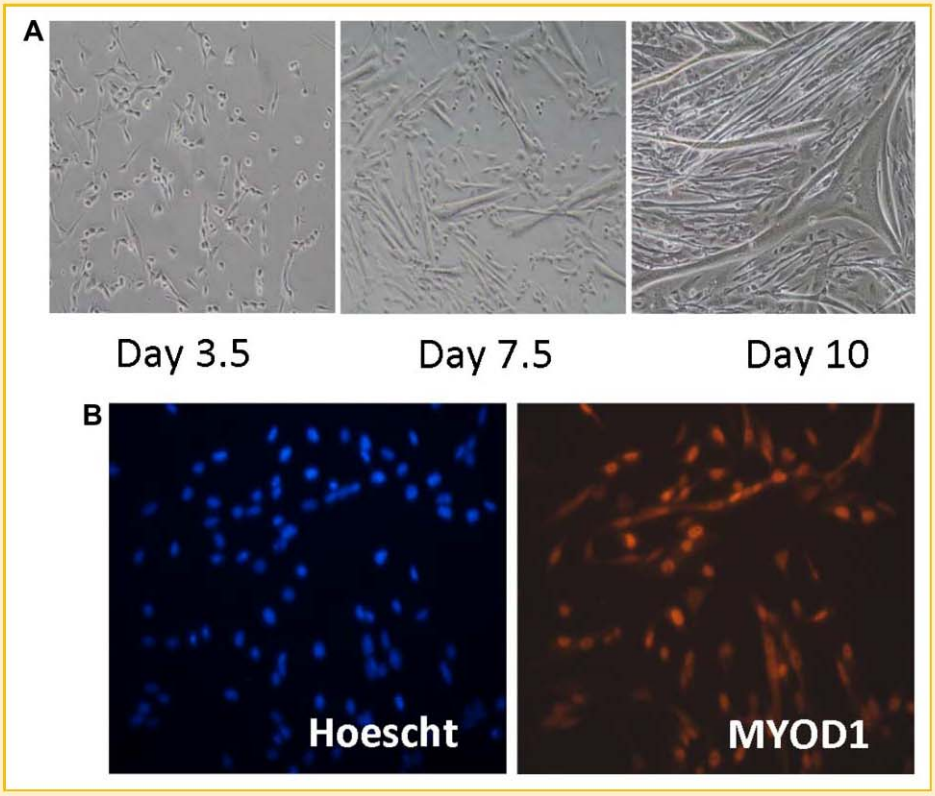

Fig. 1. Proliferation and differentiation process of mouse myogenic cell cultures. A: Appearance of cultures 3.5, 7.5, and 10 days postplating. B: Myogenic origin of the cells was assessed 4.5 days postplating by co-staining for Hoechst and immunocytochemistry for MYOD1. The percentage of cells that expressed MYOD1 at this stage was 70–80%.

5.5 where proliferation was prominent without significant differentiation process and at day 7.5 where clear differentiation into myotubes was observed. In myoblasts from 7-day-old mice, no significant differences between sexes were found in *Myod1* expression at day 5.5 of culture in EDL (Fig. 6A) or SOL (Fig. 6B), and the same was true for day 7.5 of culture (Fig. 6C and D, respectively). However, starting from age of 40 days and up till 120 days, more actively proliferating male myoblasts expressed generally higher levels of *Myod1* than females in both EDL and SOL muscles (Fig. 6A–D, see Supplementary Table 2 for statistics). The same was observed consistently in days 5.5 and 7.5 of cell culture, despite the fact that in one point this did not reach significance (Fig. 6C, 60 days of age, $P = 0.094$), and in 120-day-old *EDL* females actually expressed more *Myod1* (Fig. 6C, $P = 0.002$). Despite this odd data point, these results are generally consistent with proliferation data that suggests increased activation and proliferative potential of male satellite cells commencing around 40 days of age. Therefore, contrasting results obtained from rats may represent true differences between these two rodents or, alternatively, derive from the use of extracellular matrix components (Matrigel) in our study compared with gelatin by Lagord et al. (1998).

Differentiation of myoblasts in vitro is regulated by many factors, including cell proliferation, migration to establish cell–cell contacts and cell fusion (Seale and Rudnicki, 2000). Proliferation rate in restricted space, such as cell culture well, is inherently related to the cell density because faster proliferating cells will reach confluence sooner. Myoblasts are known to migrate towards developing myotubes which enhances the fusion process and, therefore, facilitates differentiation. Assuming no differences in differentiation potential per se (e.g., by aberrant cell cycle regulation) one would expect higher proliferation rate to lead to increased differentiation as the cells reach critical density faster. In our conditions, differentiation process was considered prominent at day 7.5 of culture as evidenced by frequent myotube formation was (Fig. 1A). At this timepoint, *myogenin and Mrf4*, markers of differentiation process, *were* determined. In myoblast cultures from mice of 7 days of age, *myogenin* expression was only significantly ($P = 0.039$) higher in EDL in females compared to males (Fig. 7A) consistent with a increased proliferation in females at this age (Fig. 2A). Expression of *myogenin* in SOL-derived cells, as the proliferation rate, was not affected at this age (Fig. 7B, $P = 0.493$). In contrast, Mrf4 was not affected in EDL (Fig. 7C, $P = 0.429$) and was downregulated in SOL (Fig. 7D, $P = 0.014$). In 40 days of age, where *Myod1* was upregulated in both muscles of males, we found consistent increase in *myogenin* expression in male EDL ($P < 0.001$) and SOL ($P = 0.038$) (Fig. 7A–D, respectively), Mrf4 was also

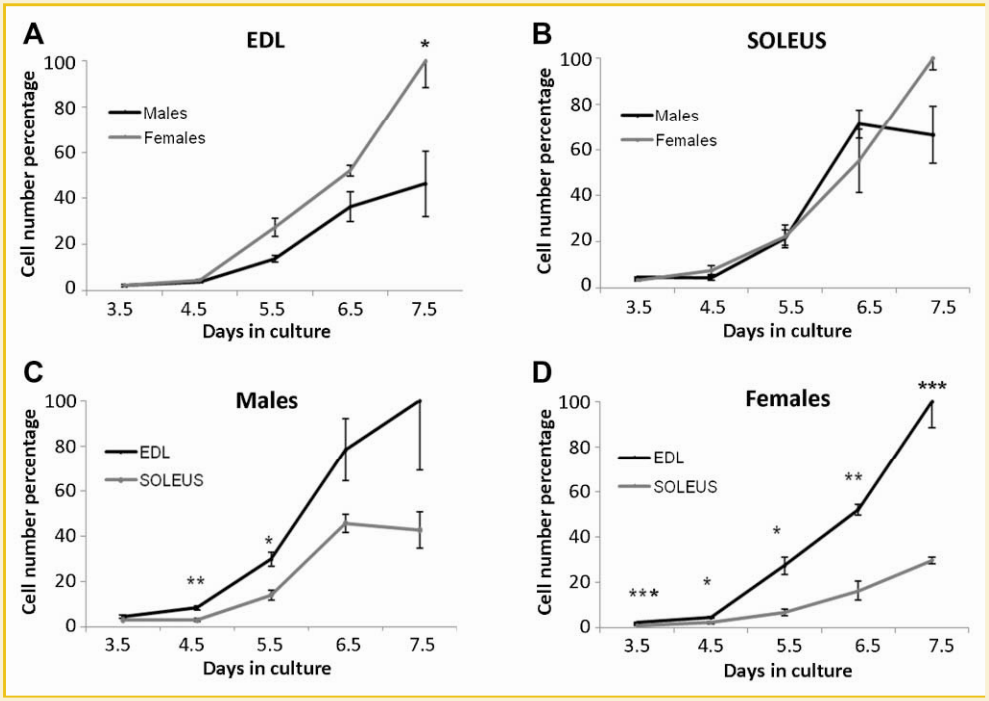

Fig. 2. Myoblast proliferation curves at 7 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or < 0.001 (***).

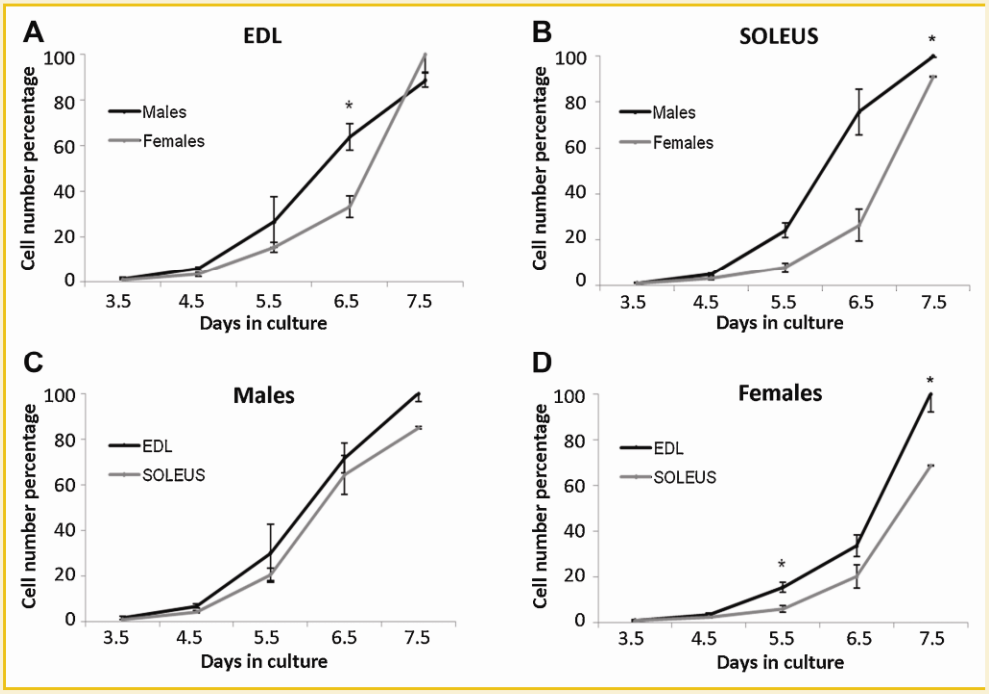

Fig. 3. Myoblast proliferation curves at 40 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*).

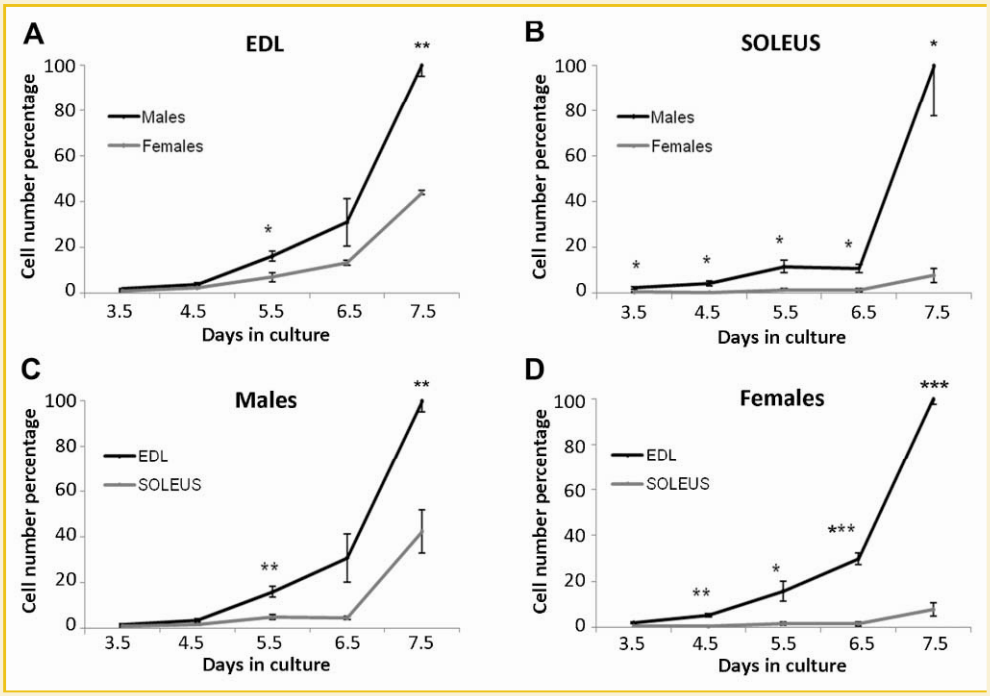

Fig. 4. Myoblast proliferation curves at 60 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or < 0.001 (***).

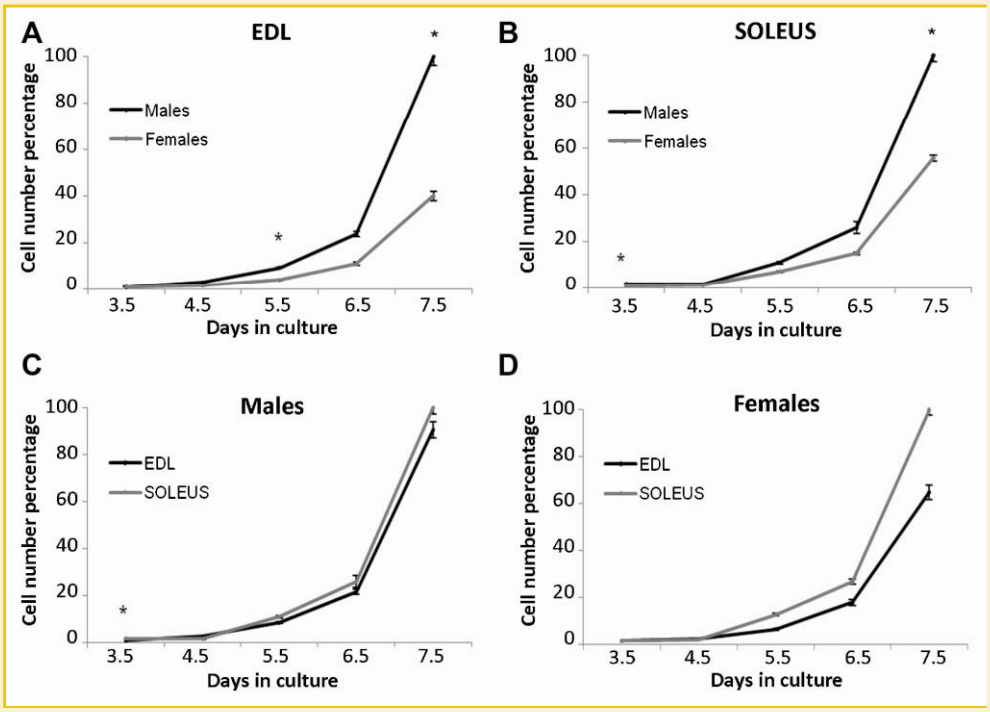

Fig. 5. Myoblast proliferation curves at 120 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*).

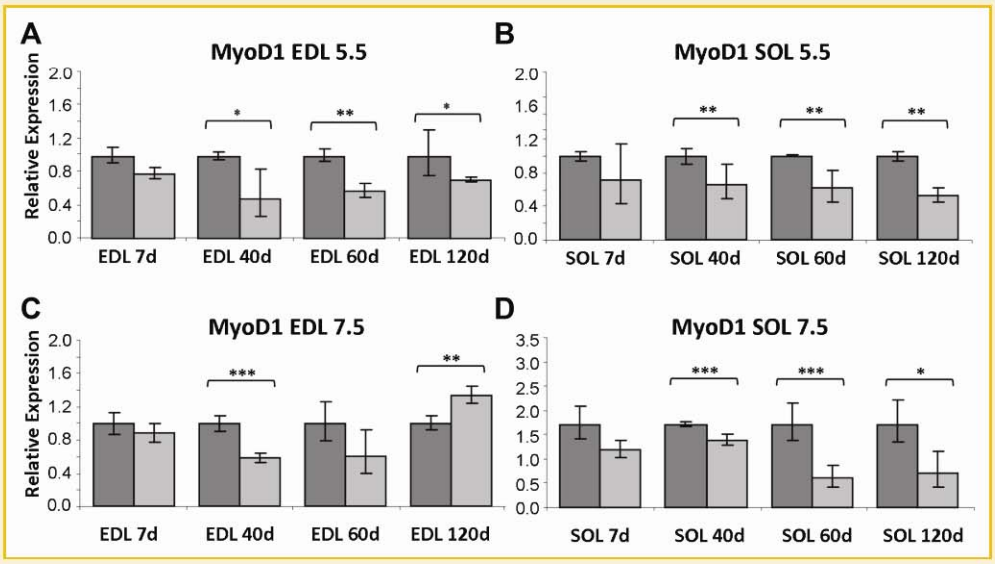

Fig. 6. *Myod1* expression in proliferating and differentiating myoblasts. *Myod1* transcript levels in male (dark gray bars) and female (light gray bars) myoblasts from mice of age 7, 40, 60, and 120 days, shown as relative expression compared with males at each age. (A) EDL and (B) SOL myoblasts at 5.5 days of culture. (C) EDL and (D) SOL myoblasts at 7.5 days of culture. Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or <0.001 (***).

upregulated at this timepoint in EDL (Fig. 7C, $P=0.033$) although in SOL this did not reach significance (Fig. 7D, $P=0.328$). Although both cell proliferation data and increased *Myod1* were consistent with enhanced proliferation rate in males of 60 and 120 days of age, this was not *consistently* reflected in the level of *myogenin* (Fig. 7A and B) of Mrf4 (Fig. 7C and D). These observations suggest that females older than 60 days of age, differentiation process may be *altered* in both EDL and SOL compared with males.

Pax7 is marker of quiescent satellite cells, its expression being gradually down-regulated in activated myoblasts as the differentiation process starts (Zammit et al., 2004). *Pax7* overexpression blocks myogenesis by a mechanism involving regulation of MyoD protein stability (Olguin et al., 2007) and prevents *myogenin* induction promoting cell cycle exit (Olguin and Olwin, 2004). Here, the analysis of *Pax7* expression in satellite cell/myoblasts of male and females EDL and SOL muscles at 5.5 days in culture revealed a tendency for an upregulation of *Pax7* transcripts in females compared to males. The most prominent and significant differences were found in cultures of 40 days old mice ($P<0.001$ and $P=0.004$ for EDL and SOL, respectively), the tendency was small but consistent in most ages under study and in both EDL and SOL muscles (Fig. 7E and F). The drastically decreased Pax7 expression in males relative to females at 40 days of age is consistent with enhanced activation of male satellite at this age as measured by increased expression of *Myod1* (Fig. 6), *myogenin and Mrf4* (Fig. 7).

DISCUSSION

Distinct characteristics that depend on sex, muscle type, and age are frequently reported in skeletal muscle or muscle derived stem cells, which highlights their importance as a possible source of biological variation (Lagord et al., 1998; Velleman et al., 2000; Mesires and Doumit, 2002; Huang et al., 2006; Deasy et al., 2007). Especially gender-dependent variation deserves consideration when new innovative approaches for experimental or therapeutic protocols are established since, even if unintentional, sex-biased basic research may lead to qualitatively different and possibly risky interpretations (Check Hayden, 2010). Whereas cross comparison of studies using different species may already be difficult, additional level of complexity derives from various isolation methods and cell culture techniques carried out in different laboratories. While species-, gender-, or muscle-specific information from different laboratories can actually provide complementary or contrasting information that drives research forward, simultaneus studies of satellite cell proliferation comparing above mentioned parameters are rarely reported. Additionally, studies describing the age-dependent differences in muscle function are largely comparisons of young, old, and senile animals whereas less is known about modulation of muscle stem cell properties during early postnatal development, although two recent studies have elegantly addressed this issue lately ex vivo in mouse EDL (White et al., 2010) and in vitro in pooled hind-limb muscles of male rats (Suzuki et al., 2010). This is important because most research carried out on muscle stem cells utilize material from relatively young animals, where possible complications with respect to postnatal muscle growth, hormonal status are largely undefined.

By providing myonuclei to postnatally growing muscle fibers satellite cells are essential building blocks for construction of adult musculature. In mice the number of myonuclei reach their adult levels by 3 weeks of postnatal life after which the muscle growth reflects solely increased cytoplasmic volume without alteration in

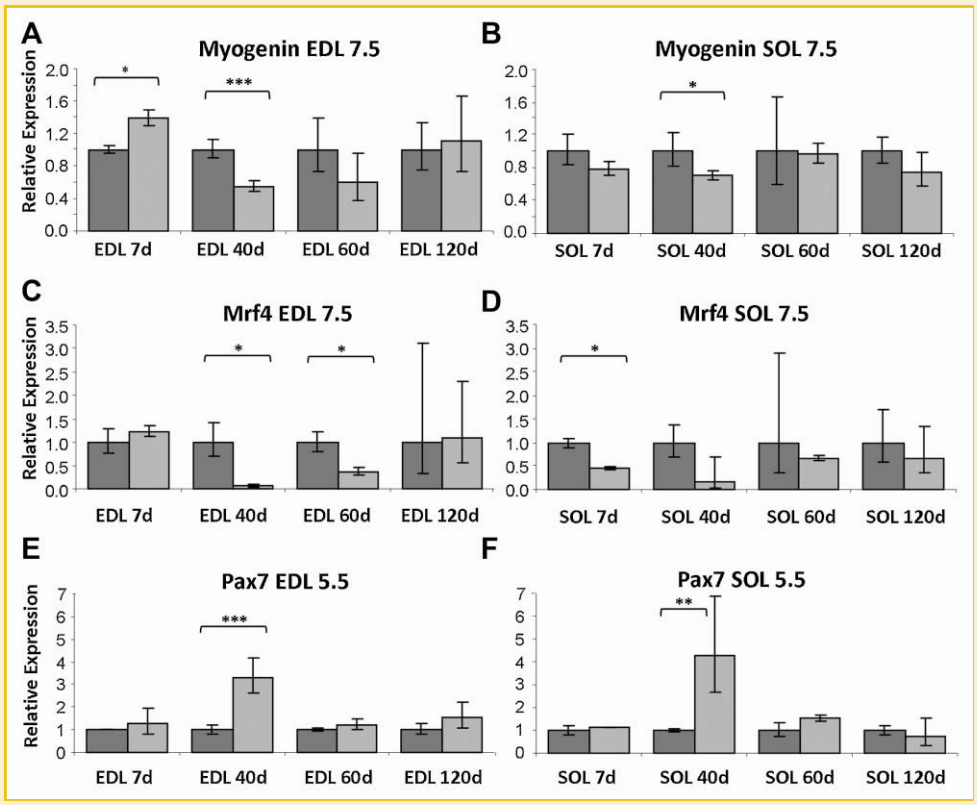

Fig. 7. *Myogenin*, *Mrf4*, and *Pax7* expression in myoblast cultures. Transcript levels in male (dark gray bars) and female (light gray bars) myoblasts from mice of age 7, 40, 60, and 120 days, shown as relative expression compared with males at each age. *Myogenin* expression in differentiating (A) EDL and (B) SOL myoblasts at 7.5 days of culture. *Mrf4* expression in differentiating (C) EDL and (D) SOL myoblasts at 7.5 days of culture. *Pax7* expression in proliferating (E) EDL and (F) SOL myoblasts at 5.5 days of culture. Error bars represent mean ± standard deviation converted to linear scale (Materials and Methods Section). Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or < 0.001 (***).

the number of myonuclei (White et al., 2010). Consistently, satellite cell number in EDL reaches its adult level by age of 3 weeks (White et al., 2010) at which point their metabolic activity declines and they exit from the cell cycle. Although these results clearly imply that determinants of adult muscle in mice are defined in early postnatal life, similar studies have not been carried out yet in slow-twitch muscle, and the sex-dependency, if any, has not been adressed.

In the present work we have described in vitro proliferation potential and MRF transcript levels in mouse satellite cell-derived cultures of the two genders, in EDL and SOL muscles from individuals at 7, 40, 60, and 120 days of age. We found that myoblast proliferation rate of juvenile (7-day-old) mice is higher in fast-twitch EDL of females, whereas that of SOL-derived myoblasts is not affected by sex. However, male myoblasts show increased proliferation rate starting from 40–60 days of age and continuing at least till 4 months of age. In contrast to what has been found in male rats (Lagord et al., 1998), EDL-derived satellite cells from male and female mice generally proliferate faster in vitro that those from SOL muscle, although this difference is possibly abolished in more advanced age. The sex-specific proliferation data is supported by general increase in *Myod1* expression in actively proliferating male-derived cells. However, the expression of *myogenin and Mrf4* is not consistently higher in males, but is only significantly up in EDL and SOL of 40-day-old males. At this point, female cells also show greatly increase *Pax7* expression compared with males, which is consistent with the described role of *Pax7* to inhibit myogenesis and cell cycle progression (Olguin and Olwin, 2004) and suggests enhanced capacity to activate quiescent cells in male-derived cells.

These results are partially consistent with the fact that sex hormones may influence satellite cell proliferation and MRF expression. Androgen receptors (Sinha-Hikim et al., 2004) and estrogen receptors (Guo et al., 2010) are expressed in satellite cells. Testicular steroidogenesis in male mice increases dramatically between 30 and 60 days in mice and could possibly explain the lower proliferation rate in 7-day-old males, when testosterone levels are still low. Testosterone stimulation can induce *Myod1* expression in cultured multipotent mesenchymal cells (Singh et al., 2003) whereas *removal* of estrogen by ovariectomy causes similar increase in muscles of female mice (Rogers et al., 2010). Although these studies may *suggest* that male and female sex hormones have opposite effects on MRF expression, effects of testosterone on in vitro myoblast proliferation are contrasting (Chen et al., 2005) and

also positive effect of estrogen on satellite cell activation has been described (Enns and Tiidus, 2008). In any case, in conditions used in our study it seems unlikely that the potential hormonal effects would be persistent enough to carry out their influence through several days of in vitro culture.

Increasing evidence suggests that gender differences in satellite cell function may not be hormonal but innate. In an extensive study carried out in vitro and in vivo using mouse model of Duchennés muscular dystrophy (mdx), muscle-derived stem cells from females exhibited higher regenerative potential compared to males (Deasy et al., 2007). Based on enhanced tendency of male derived cells to differentiate under oxidative stress in vitro, it was proposed that male and female cells may also exhibit distinct response to oxidative stress induced by muscle injury in mdx mice. In this model, male cells would rapidly activate, proliferate, and differentiate into myotubes whereas female cells would be less proliferative, being able to perform more rounds of proliferation and ultimately differentiate, fuse, and regenerate muscle effectively when acute inflammation is over (Deasy et al., 2007). Alternatively, female satellite cells may have better survival when transplanted if their expression of *Myod1* is lower than that of males, as satellite cells lacking *Myod1* show increased survival when transplanted to regenerating muscle (Asakura et al., 2007). Although these studies are consistent with our findings with respect to enhanced proliferation rate and *Myod1* expression of male cells in 40–120 days of age, as well as with increased *Pax7* expresion in 40-day-old females, Deasy et al. (2007) reported no gender differences for in vitro proliferation in the absence of oxidative stress. Several possible reasons could explain the differences, such as different muscle used, different method used to extract muscle derived stem cells, and different age of animals (21 days of age in Deasy et al., 2007). Although we did not study mice at age of 20 days, it is worth repeating that no consistent sex-dependent effects on MRF expression was observed in juvenile mice (7 days of age), and the effect on proliferation was muscle-dependent.

Clonal analysis carried out recently (Rossi et al., 2010) indicates that satellite cells, even those derived from the same muscle fiber, may exhibit intrinsic differences in their ability to proliferate and differentiate. It was found that cells proliferating at low rate (LPC) spontaneously generate myotubes, whereas highly proliferative cells (HPC) may either differentiate into adipocytes or, when co-cultured with LPCs, eventually form myotubes. In this study, we cannot exclude that satellite cells from females and males may consist of uneven proportions of LPCs and HPCs. Higher proportion of HPCs in males could potentially explain their rapid rate of in vitro expansion. If male cells indeed have higher proportion of HPCs, which are likely to be more sensitive to oxidative stress (Rossi et al., 2010), this would possibly explain why oxidative stress in vitro would favor differentiation of male cultures (Deasy et al., 2007). When transplanted, HPCs also show lower regenerative potential in vivo (Rossi et al., 2010), consistent with lower regeneration potential of muscle-derived stem cells from males (Deasy et al., 2007). Although we cannot prove causality in our studies, these observations may suggest higher proportion of HPCs in male muscles, which is warrant further studies. If this is the case, however, differentiation of these cells is not negatively affected in our conditions as no gross defect in *myogenin* and *Mrf4* induction is observed. The possible sex differencas in LPC/HPC content warrant further studies in the future.

The comparison of the myoblast proliferation rate between the two muscle types indicated that cells from EDL, formed mainly of fast-twitch muscle fibers, proliferate faster than cells from SOL, consisting of slow-twitch fibers. This result was true from juvenile (7-day-old) to young, sexually mature mice (60-day-old) in both sexes. However, the situation was reversed in older mice, especially in females where SOL proliferated faster than EDL at age of 120 days. Fast and slow muscle-derived satellite cells have been shown to differ in physiology and, in vitro, are imprinted to produce muscle fibers with distinctive characteristics of their fibers of origin (Huang et al., 2006), that is, muscle fibers engineered in vitro from slow muscle satellite cells contract and relax slower than similar tissues engineered from the fast muscle. This demonstrates that satellite cells from slow and fast-twitch muscles are intrinsically different. Hence, it is not unreasonable that their ability to proliferate and differentiate in vitro is different. Fast-twitch muscle fibers are more abundant than slow-twitch in adult skeletal muscle. It could be hypothesized that during postnatal muscle growth when fast-twitch fiber supply is needed more to increase muscle bulk, cells derived from fast muscle would be capable of proliferating at a higher rate to build up muscular tissue. By contrast, adult slow-twitch fibers posses higher number of satellite cells than fast-twitch fibers (Hawke and Garry, 2001), probably because the first and most frequently recruited muscles during every day activity are slow-twitch type (Hawke and Garry, 2001; Kadi et al., 2006). These data correlate to our finding that 120 days old SOL satellite cell cultures proliferate at similar (males) or higher rate (females) compared to EDL cultures, thus being capable of responding to typical requirements of adult muscle activity. It is clear that in vitro studies may not perfectly reflect satellite cell dynamics in situ, where their function is likely to be modified by complex interactions in their myofibrillar niche. However, positive switch in relative proliferation capacity of SOL-derived cells in more advanced ages is consistent with relatively higher amount of satellite cells in slow compared with fast muscle in adults.

Gender and muscle type-dependent variation in proliferation and MRF expression were modulated by postnatal age of the animal, perhaps suggesting that satellite cell properties, or relative abundance of high and low proliferating cells, may experience dynamic changes in growing postnatal muscle. In very young mice, no gender differences were found in proliferation and differentiation process in cells from slow-twitch SOL muscle. However, increased cell proliferation and *myogenin* (but not *Myod1*) expression was found in EDL-derived cultures from juvenile females. It is unknown why *Myod1* was not upregulated in these faster proliferating female cells. One potential explanation is that, as discussed above, these results may reflect higher proportion of spontaneously differentiating cells (Rossi et al., 2010) in *juvenile* females. In satellite cell cultures from 40-day-old mice, higher levels of *all* MRFs were found in males than in females, in both EDL and SOL muscles (although Mrf4 in SOL was only suggestive). Indeed, in this postnatal age the sex-dependent effect on MRF expression were most consistently male biased, and increased *Myod1* and *myogenin*

expression was correlated with relatively decreased *Par7* expression. Collectively, this suggests depletion of quiescent satellite cell pool in fastly proliferating and differentiating male cells. This result is in concordance with higher in vitro myoblast fusion index in males compared to females in 7-week-old turkeys (Velleman et al., 2000). Male cells continued to generally express higher levels of *Myod1* in myoblasts derived from 60 to 120-day-old muscles (except in EDL 7.5-day culture). However, *myogenin* and Mrf4 were not consistently affected in EDL or SOL, although relatively high variation in the replicate cell cultured prevented significance in some cases. Increasing the number of replicates was not feasible because of high number of variables investigated (sex, age, and muscle type), and to avoid variation in the cell quality the priority was set to extract and process both muscles and both genders at the same time. Nevertheless, realtively lower *Myod1* in females coupled with similar levels of *myogenin* as in males may suggest that female cells may be prone to differentiate earlier in vitro. This early differentiation is not likely to depend on cell density as females at this stage proliferated much slower than male cells. These results encourage further research on potential differences on cell cycle regulators, as well as detailed characterization of differentiation capacity in different sexes and muscle types.

To our knowledge, this is the first study investigating the specific influence of muscle fiber-type muscle, sex, and postnatal age in the same experimental and cell culture conditions. We have concluded that male satellite cells exhibit higher proliferation rate compared to females in EDL and SOL muscles from young to adult mice, and in EDL muscle compared to SOL in both sexes from juvenile to young mice. The proliferation rates are generally reflected in the expression of MRF genes, although these changes are modulated by postnatal age of the animals. The results presented strongly support the notion that the satellite cell heterogeneity is not only externally induced but also an intrinsic character of these cells. Therefore, our study remarks the importance of gender, muscle type, and age as important factors to understand postnatal muscle growth and regeneration. These results may have special importance for research on regenerative medicine of muscle or neuromuscular disorders, where the source of the regenerative cells has to be appropriate to ensure the efficacy of the satellite cell-mediated therapy. Recently, effort has been taken to transplant intact or genetically modified satellite cells to alleviate neuromuscular disease in animal models (Lee et al., 2000; Montarras et al., 2005; Deasy et al., 2007). Therefore, our results indicate that, to ensure unbiased interpretation before clinical application, further work should be carried out preferably in the two sexes and, if possible, using muscle types closely resembling those of the target tissue.

ACKNOWLEDGMENTS

We apologize for those authors whose relevant work was not cited due to size restrictions. We thank María Royo and Mamen Carreras (Microscopy and Image Service) for technical assistance with fluorescence microscope, and the I+CS (Aragon Health Sciences Institute) for access to the microscope. The study was supported by grants CAJA NAVARRA: "Tú eliges, tu decides"; PI071133 from the Fondo de Investigación Sanitaria of Spain and PAMER from the Instituto Aragonés de Ciencias de la Salud (PIPAMER 09/09).

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Housekeeping gene expression in myogenic cell cultures from neurodegeneration and denervation animal models.

Manzano R, Toivonen JM, Calvo AC, Muñoz MJ, Zaragoza P, Osta R.

Biochem Biophys Res Commun. 2011 Apr 22;407(4):758-63

Biochemical and Biophysical Research Communications 407 (2011) 758–763

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Housekeeping gene expression in myogenic cell cultures from neurodegeneration and denervation animal models

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ARTICLE INFO

Article history:
Received 15 March 2011
Available online 31 March 2011

Keywords:
Neurodegeneration
Denervation
Muscle
Real-time PCR
Housekeeping

ABSTRACT

Reliability and accuracy of real-time quantitative PCR results depend on the use of housekeeping genes which must be constitutively expressed thorough the samples of the study. In the present work, we tested the expression stability of six candidate housekeeping genes (*Actb, Rn18s, Gapdh, Hprt*1, *Sdha and B2m)* considering sex, age, muscle-type and neurodegeneration or denervation status in mouse muscle satellite cells. Their expression varied under all variables tested; therefore the ranking of the most suitable genes for the normalization is modified depending on the factors included in the analysis, especially the age of the donor. Moreover, we describe the unsuitability of *Rn18s* in analysis comprising samples of different ages. On the other hand, we demonstrate that the use of the two best genes in each case is enough to obtain a reliable normalization factor. In this work, we give a broad information of the best housekeeping genes in mouse myogenic cells depending on the variables included in the experimental design.

1. Introduction

Satellite cells are muscle-derived progenitors located beneath the basal lamina of mature skeletal muscle fibers. Since the first description by Mauro in 1961, these cells have been the target of numerous studies in regenerative medicine and tissue engineering [1–3]. Culturing and differentiating muscle satellite cells *in vitro* has allowed researchers to study these cells under several conditions, including pathological processes and therapeutic approaches [4–6].

Many studies of the characteristics and changes of satellite cells cultured *in vitro* are performed by gene expression analysis and quantification using real-time quantitative PCR (qPCR) [7,8] relying its accuracy results on normalization to an internal control, often referred to as a housekeeping gene [9,10], whose expression must be constitutive and able to be analyzed by qPCR in the same sample as the target gene [11,12]. However, reference gene expression stability must be validated because results derived from the normalization with different reference genes have been shown to vary [13,14]. For this purpose geNorm software has been widely used [15].

Particularly, in skeletal muscle tissue, housekeeping gene expression has been shown to be affected by age [16], acute exercise [17,18] or muscle fiber type [17]. By contrast, the analysis of typically used housekeeping genes in myoblast cell cultures has rarely been studied. Only two works exist, to our knowledge, using Atlantic salmon myogenic cell cultures and human myoblasts [19,20]. In any of these cases, the sex of the patients was reported, or comparisons between different ages or muscles types were carried out. No studies have been performed in mouse skeletal muscle satellite cell cultures regarding these above-mentioned parameters. Here we present a broad analysis of the stability of six candidate housekeeping genes in both male and female mice of varying ages and in distinct fiber types, using murine differentiating wild-type and neurodegeneration models (SOD1-G93A) of myogenic cell cultures.

2. Materials and methods

All experimental procedures were approved by the Ethics Committee of the University of Zaragoza (covered by the Animal License Number 502970012007) and followed the international guidelines for the use of laboratory animals.

2.1. Satellite cell extraction and culture

Three male and female B6SJL and SOD1-G93A strain mice (model for amyotrophic lateral sclerosis) per age group were sacrificed at 7, 60, 90 and 120 days of age and EDL and soleus muscles were collected, minced to a slurry and digested in a solution containing 0.1% trypsin and 0.1% collagenase (w/v) (Sigma–Aldrich). The supernatant was collected, filtered through a 70-μm diameter ster-

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0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.03.096

ile strainer and centrifuged at 500g for 15 min. About 1000 cells per well were seeded in 96-well plates covered with 0.1 mg/mL of Matrigel (Becton Dickinson SA) and 39% F-12, 39% DMEM, 10% fetal calf serum (Gibco) and 2% Ultroser G (Pall-Biosepra). The cells were cultured for 7.5 days at 37 °C and 5% CO_2. At least two replicate wells were plated for each group. Control plates were seeded and left to proliferate and differentiate for 10 days for analysis of correct differentiation and myotube formation.

2.2. Muscle denervation procedure

Following anaesthesia, six male and six female 60 day-old mice (B6CLJ strain) were denervated unilaterally in the right leg by extracting a 5 mm segment of the sciatic nerve. The left limb was used as a control and was not denervated. Fifteen days after surgery, animals were sacrificed, and control and denervated EDL and SOL muscles were microdissected and processed for satellite cell extraction and culture.

2.3. Immunocytochemistry

To ensure the myogenic origin and correct differentiation of the growing cells, after 7.5 days of culture, one control well per group was fixed and immunostained for the satellite cell differentiation marker Myogenin. Cells were rinsed, fixed in 4% neutral buffered formalin (Sigma–Aldrich), permeabilized with 0.2% (v/v) Triton X-100 (Sigma–Aldrich) and blocked using 0.2% (w/v) gelatine (Sigma–Aldrich, Madrid, Spain). Antibody to Myogenin (1:200), was added for 2 h (Clone F5D, Dako) and revealed with anti-mouse secondary antibody (Invitrogen) before mounting in a solution containing 1000 ng/mL Hoechst 33342 (Sigma–Aldrich). Cells were visualized under a fluorescence microscope.

2.4. RNA extraction and reverse transcription (RT)

The mRNA extraction and the cDNA synthesis were performed using the Cells-to-cDNA kit (Ambion) according to manufacturer's instructions. The cells were lysed in 100 μL of ice-cold cell lysis solution, followed by heat treatment at 75 °C for 15 min. 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. Two cDNA synthesis reactions from each replicate well were performed. Briefly, 2 μL of dNTPs and 1 μL of random hexamers were mixed with 5 μL of RNA, followed by incubation at 75 °C for 3 min. Two microliters of master mix composed of 1 μL of 10× reverse transcription buffer, 0.5 μL of M-MLV reverse transcriptase and 0.5 μL of RNase inhibitor were added and incubated at 42 °C for 60 min and 95 °C for 10 min.

2.5. Quantitative real-time PCR (qPCR)

For qPCR, reactions were performed in triplicates in a final volume of 5 μL; using 2 μL of cDNA diluted 20-fold with dH_2O, 2.5 μL of Fast 2× TaqMan master mix and 0.5 μL of gene-specific TaqMan assays (Applied Biosystems) (see *Supplementary* material File 1). Reactions were run using the StepOne Plus Real-Time PCR System (Applied Biosystems) using the manufacturer's conditions. All reactions had a PCR efficiency value (E) of 2 ± 0.03 approaching 100%. To normalize data for geNorm, C_t values were transformed to relative gene expression values using the equation $E^{\Delta C_t(C_t\,\mathrm{min}-C_t\,\mathrm{sample})}$ [21].

2.6. GeNorm analysis

Relative gene expression values were plotted for geNorm stability analysis, obtaining an M index for each gene in each single comparison. These values represent the average pairwise variation for that gene compared to all other tested reference genes. Higher M values represent lower stability for that gene within the group of candidate reference genes. For the calculation of the minimum number of genes necessary for an accurate normalization factor, the pairwise variation, V, between two consecutive normalization factors containing an increasing number of genes was assessed [21].

2.7. Statistics

Comparisons among distinct groups were carried out using Student's t-test (Statistic 5.0). Statistical differences were considered significant at $P < 0.05^{*}$ or 0.01^{**}. Student's t-test values $P < 0.1$ are shown as black arrows as they are very close to significance.

3. Results

First, we verified that our samples truly represented differentiating satellite cells by immunohistochemical staining for Hoechst and Myogenin (Supplementary material File 2, C and D) a well-known satellite cell differentiation marker, in 7.5-day cultures [22–24]. Moreover, control 96-well plates were left to proliferate and differentiate for 10 days to observe the formation of fully developed myotubes (Supplementary material File 2, A and B).

3.1. Housekeeping gene expression during postnatal development and in adults

In comparisons including samples of different ages, all six genes showed mean M values above 1.2 with a mean of 1.292 ± 0.016, which highlights the substantial influence of age on the variation of housekeeping gene expression. In fact, *Rn18s* displayed an M value higher than 1.5, the threshold to be considered by geNorm as a suitable housekeeping gene (see *Supplementary* material File 3 for complete data). Moreover, when determining the minimal number of housekeeping genes recommended for the correct normalization for these conditions, all six genes were included by geNorm (*Supplementary* material File 3). To quantitatively analyze the influence of age on the variability of the studied housekeeping genes, M values of each gene were averaged for each group by sex, muscle type and phenotype, first by considering every age separately and then comparing these values to those obtained when pooling all ages for each factor (*Supplementary* material File 3). This analysis showed a significant increase in the M average when pooling samples of different ages ($P < 0.05$ or 0.01), and this increment was consistent for sex, muscle type or phenotype (Fig. 1A) and when considering these factors together (Fig. 1B).

3.2. Influence of sex, muscle type and neurodegeneration progress on housekeeping gene expression, intra-age analysis

Hereafter, to avoid the disguising effect of age, the analyses were performed on each age separately, to distinguish the effects of sex, muscle type and phenotype on the expression of the genes of interest. Our comparisons revealed that geNorm-based ranking of the most to the least stable genes was different for each group. Hence, all of the factors under study influenced, to some degree, the expression of the candidate reference genes. However, all of these comparisons displayed M values below those obtained in the first analysis, with an M total average of 0.622 ± 0.04 (*Supplementary* material File 3). Consequently, all of the values were below the geNorm limit of 1.5 and were suitable to be used as reference genes in real-time PCR studies. In these conditions, the analysis of the minimum number of genes recommended to calculate the normalization factor showed that, when considering all factors for each age, two genes would be enough for cultures of 7

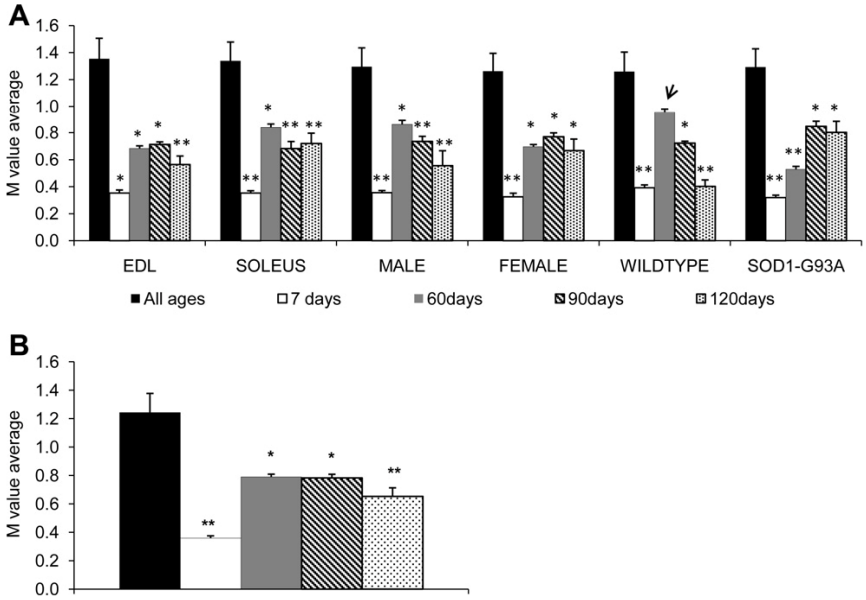

Fig. 1. GeNorm *M* values average comparison. Representation of the six housekeeping genes *M* values average for each group and age (A) and general (B). Student's *t*-test comparisons were carried out for each group among each age and all ages *M* average. Black arrows $P < 0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$.

and 120 day-old animals, but four genes would be necessary when considering 60 and 90 day-old animals because the latter two ages seem to present the highest variability. When comparing the distinct groups of each age, two housekeeping genes were enough in most cases (*Supplementary* material File 3).

3.3. Effect of normalization factor on the analysis of expression of a gene of interest

For each group we normalized *Pax7* expression, a satellite cell marker, to each housekeeping gene separately and to the geometric average of the number of genes recommended by geNorm analysis for each age. Subsequently, we compared the expression of our target in each group, setting extensor digitorum longus (EDL), male and wild-type group to 1 as calibrators, and determining soleus (SOL), females and SOD1-G93A *Pax7* expression, respectively, as a fold-change. In 7 day-old mice satellite cell cultures, no differences were found in terms of *Pax7* expression between control and target groups whatever normalization factor was used (Fig. 2A). By contrast, in 60 day-old mice, female cells displayed lower levels of *Pax7* transcript than males if normalizing to *Rn18s*, *Actb* or the normalization factor suggested by geNorm ($P < 0.05$) but not with *Gapdh, Sdha, B2m* or *Hprt1* themselves. In muscle type comparison, soleus samples showed increased expression of *Pax7* when normalizing to *Rn18s* ($P < 0.5$), and with *Gapdh*, the Student's *t*-test value was close to significance ($P < 0.1$). Normalization to the rest of the housekeeping genes or to the geNorm-suggested factor revealed no significant differences between EDL and soleus cells or even a tendency to lower *Pax7* levels in soleus that, as in the case of normalization with *B2m*, was almost significant ($P < 0.1$). Analysis of wild-type and SOD1-G93A satellite cell cultures revealed no differences with any of the references used (Fig. 2B). In 90 day-old cultures, no differences were found in terms of *Pax7* transcript levels between males and females with any of the normalization factors. However, a significant decrease was found in soleus compared to EDL when normalizing to *Hprt1, Sdha* or *B2m* independently or to the geNorm-suggested factor ($P < 0.05$). Using *Rn18s, Gapdh* and *Actb*, the tendency was similar, although not significant. Similarly, a decrease was found in SOD1-G93A compared to wild-type when normalized to *Rn18s*, *Gapdh*, *Actb* ($P < 0.01$), *Hprt1*, *B2m* ($P < 0.05$), *Sdha* ($P < 0.1$) and geNorm factor ($P < 0.01$) (Fig. 2C). In 120 day-old cultures, no differences were found in *Pax7* relative expression by sex or muscle type with any of the normalizations assayed, (see *Supplementary* material File 3). However, an increase of *Pax7* gene expression was observed in SOD1-G93A with every reference gene tested as well as with the geNorm factor, although these values are only significant in the case of *Actb* ($P < 0.05$) and *B2m* ($P < 0.01$) (Fig. 2D).

3.4. Stability of housekeeping genes in satellite cell cultures from long term denervated muscles; effect of normalization factor

The results showed low *M* values in comparisons including denervated and control groups with an average of 0.343 ± 0.013 (*Supplementary* material File 4). Again, there is a certain degree of influence of sex and muscle type on the expression stability of these genes. Hence, the geNorm ranking from the most to the least stable gene in each group of sex, muscle type and phenotype is not coincident, although all of them remain below the threshold of 1.5 and are therefore suitable as endogenous references. In this case, geNorm normalization factors would be composed of two of these genes, when all of the factors are included, as well as when individually analyzed (*Supplementary* material File 4). In the analysis of the normalization factor effect on the *Pax7* gene expression results we observed a clear tendency toward *Pax7* increment in denervated cultures form EDL and soleus, although except for soleus cultures normalized to *Hprt1* ($P < 0.05$), neither of the comparisons reached significance (Fig. 3A). The results was consistent for the male and female analysis where the normalization of male cultures to *Gapdh*, *Sdha* or *B2m* ($P < 0.05$) or to *Actb*, *Hprt1* or geNorm factor ($P < 0.01$) reached significance. Similarly, the denervated female group also presented a tendency towards higher levels of *Pax7* transcript with all reference genes assayed, although not significant (Fig. 3B).

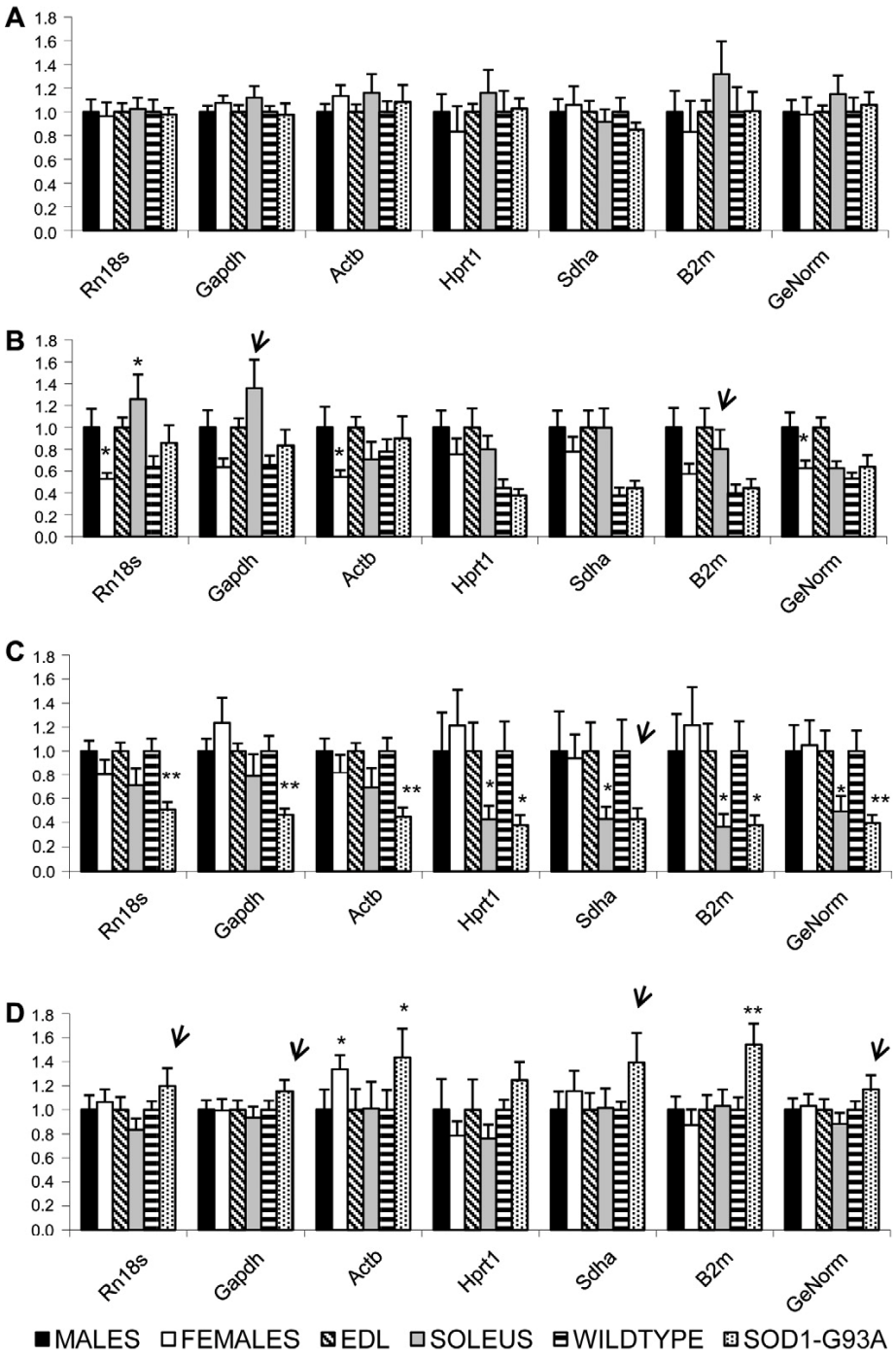

Fig. 2. Normalization factor effects on *Pax7* gene expression results. Effects of normalizing with each housekeeping gene or the combination of the best recommended by geNorm (see *Supplementary* material File 3) on the expression of *Pax7* in 7 day (A), 60 day (B), 90 day (C) and 120 day-old mice satellite cell cultures (D). Black arrows $P < 0.1$; *$P < 0.05$; **$P < 0.01$ Student's *t*-test.

4. Discussion

Despite the hundreds of publications that try to determine the best reference housekeeping gene in a particular study [13,17,21] only two of them deal with myogenic cell cultures [19,20]. Many limitations arise when their conclusions need to be extrapolated to other studies as they do not include many of the physiological variables that can influence the stability of a reference gene, such as sex, age or muscle type of the satellite cell donor [25–29]. Moreover, the inclusion of chronic neurodegenerated and acute denervated satellite cell cultures in our study extends the conclusions to a broader range of researchers that work with these kinds of

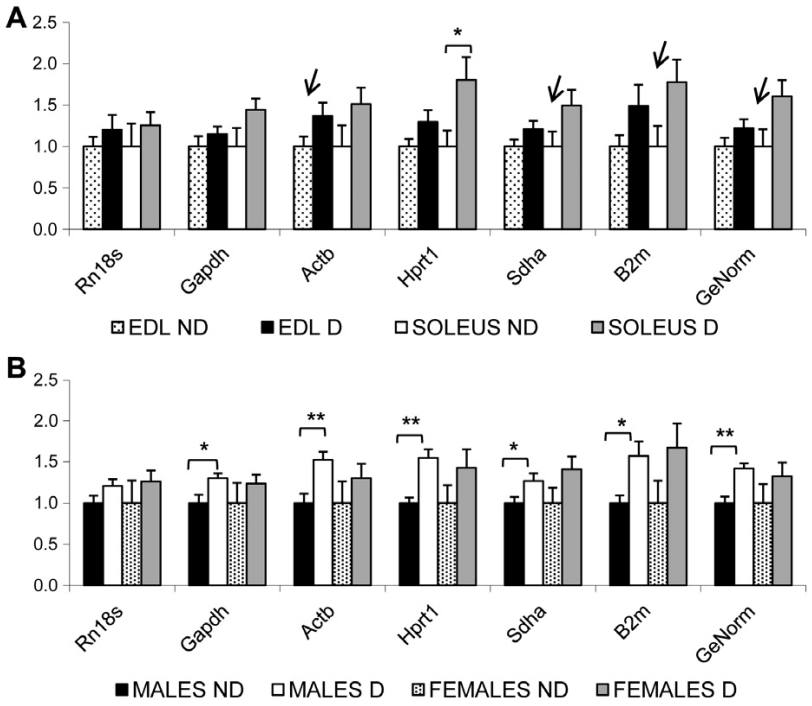

Fig. 3. Normalization factor effects on denervated satellite cell cultures: *Pax7* gene expression results. Effects of normalization with different reference genes and with the best recommended by geNorm analysis (see *Supplementary* material File 4) on the expression of *Pax7* in satellite cell cultures from denervated (D) and control muscles (ND). Comparison between EDL and soleus muscle (A) and comparison between male and female mice (B). The results are represented as fold-change (mean ± SEM) compared to the not-denervated group. Student's *t*-test comparisons are shown as black arrows $P < 0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$.

pathological conditions in their research. Overall, all factors in our study contributed to the variability of expression of the six reference genes tested. Consistently, the corresponding *M* values have a mean of 1.292 ± 0.016, very close to 1.5, the suitability threshold established by geNorm [15]. Some studies report the instability of Gapdh activity and *Gapdh, β-actin* and *B2m* mRNA levels in aging muscle [16,30–32]. In contrast, studies performed in Atlantic salmon and human myogenic cell cultures the *M* values for all of the candidate genes were below 0.5 [19,20]. However, neither of these groups performed comparisons between samples of different ages, sexes or muscle types, and their experiments were developed exclusively with healthy donors, which conform to relatively homogeneous sample groups. Furthermore, under our conditions, the *Rn18s M* value is above 1.5 and should not be included in the normalization factor calculation. This feature could be related to the fact that, as opposed to mRNA genes that are transcribed by RNA polymerase II, rRNA transcription is carried out by RNA polymerase I, and regulatory networks modifying the transcriptional activity of both enzymes, such as oxidative stress or growth factor stimulation, can be affected in a dissimilar manner [33]. On the other hand, when samples are analyzed per age group, the *M* values of all of the genes as well as the minimal number of genes necessary for the calculation of an accurate normalization factor decreases no matter what comparisons are performed by sex, muscle type, or phenotype or the combination of these factors. This fact highlights the influence of age in the stability of the reference genes under study. However, the ranking from the most to the least stable gene in each group is different even within each age, which demonstrates that there is certain degree of variability associated with sex, muscle type or phenotype. In this sense, McCurley and co-workers [14] showed significant differences between males and females in several housekeeping genes analyzed in zebrafish skeletal muscle. To assess the influence of choosing the correct reference or combination of genes, we normalized the expression of the *Pax7* satellite cell marker gene to each gene individually and to the combination of the most stable genes in geNorm analysis for all of the variables in each age. No large differences were expected as the *M* values of all of the genes were below the suitability threshold established by geNorm. Consistently, the results showed that the normalization to each gene individually displayed the same results as the geNorm factor, although in some cases, a disparity in the significance of the Student's *t*-test was observed. This result validates the use of any combination of generally two of these genes for the normalization of intra-age comparisons of qPCR results. The exception comes in 60 day-old samples when comparing *Pax7* expression in the EDL and soleus; the normalization to *Rn18s* or *Gapdh* revealed higher levels in soleus than EDL, opposite to the result when using the factor recommended by geNorm when sex, muscle type and phenotype are taken into consideration simultaneously. However, in this case, the use of four reference genes is recommended and the effect of *Rn18s* and *Gapdh* would be compensated for the two other genes included. The study of acute denervated muscle satellite cell cultures showed a tendency toward *Pax7* up-regulation in denervated vs. non-denervated samples in all groups of sex or muscle tested, and this result was consistent when normalizing to every single gene or to the combination of the two most stable, demonstrating that there is no drastic effect of denervation on the expression stability of the six candidate reference genes tested in any of the groups. Hence, the use of a combination of any two of them for normalization in real-time quantitative PCR approaches is sufficient. As in our previous analysis of non-denervated samples, the gene stability ranking was not coincident among all groups when comparisons were carried out independently, which points out a certain influence of sex

and muscle type on gene expression. In this sense, Yüzbaşıoğlu and co-workers [34] in 2010 validated the use of *β-actin* as an endogenous reference in studies of tenotomized rat soleus and gastrocnemius muscles up to 16 weeks rather than *Hprt1* or *Gapdh*. Interestingly, a distinct ratio of Pearson's correlations were found between gastrocnemius and soleus muscles, especially with *Hprt1 expression*. However, no information is available on satellite cell cultures in mouse either in healthy or denervated muscles. To our knowledge, this is the first study examining the stability of housekeeping genes in mouse satellite cell cultures. Neither sex, muscle type nor neurodegeneration or denervation stage drastically influenced the expression of the reference genes analyzed. However, the age of the donors strongly increased their variability. Thus, would advise careful consideration with the use of these genes in murine satellite cell cultures when age-dependant comparisons are needed, especially with *Rn18*. By contrast, in studies using one age, the use of the geometric average of any two of the genes is sufficient in most cases. We think that our results can serve as a database for qPCR approaches from which many researchers can extract the best reference gene depending on the variables included in their own experimental design with murine myogenic cell cultures.

Acknowledgments

This study was supported by the Grant of CAJA NAVARRA: "Tú eliges, tu decides", PI071133 and PI1001787 from the Fondo de Investigación Sanitaria of Spain and PAMER from the Instituto Aragonés de Ciencias de la Salud (PIPAMER 09/09).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.096.

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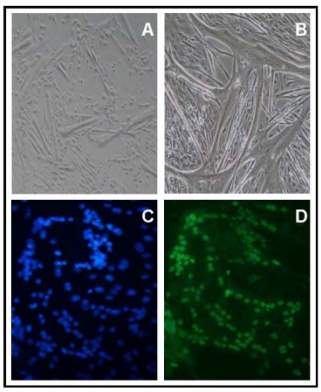
Symbol	Name	TaqMan Assay ID	Function
Rn18s	18s ribosomal rna	Hs99999901_s1	Component of the ribosome. Provides peptidyl transferase activity to tRNAs for the correct decoding of mRNAs into amino acids
Actb	Beta actin	Mm4352933E	Cytoskeletal protein involved in cell motility and structure
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	Mm4352932E	Oxidoreductase in Glycolysis and gluconeogenesis
Hprt 1	Hypoxanthine guanine phosphoribosyl transferase 1	Mm00446968_m1	Generation of purine nucleotides through the purine salvage
Sdha	Succinate dehydrogenase complex subunit A	Mm01352366_m1	Electron transporter in the citric acid cycle and respiratory chain
B2m	beta-2 microglobulin	Mm00437762_m1	Component of MHC class I molecules, participates in the immune system
Pax7	Paired box gene 7	Mm00834079_m1	Satellite cell marker

Supplementary material File 1. Taqman assay information.

Symbol[a], name[b], Taqman assay ID[c] and function[d] for each of the genes evaluated as internal controls and target *Pax7*.

Supplementary material File 2



File 2. **Satellite cell culture**
Differentiating satellite cell culture at 7.5 days (A) and 10 days (B) post-plating. Hoechst staining (C) and myogenin immunohistochemistry (D) in 7.5-day cultures.

		Housekeeping genes								
Age	**Group**	**Rn18s**	**Gapdh**	**Actb**	**Hprt1**	**Sdha**	**B2m**	**M average**	**SD**	**SEM**
All ages	**GENERAL**	1,886	**1,002**	**1,156**	**1,046**	**1,065**	**1,296**	1,242	0,332	0,136
	EDL	1,779	0.954	1,091	0.960	0.977	1,195	1,355	0,371	0,151
	SOLEUS	1,995	1,034	1,207	1,266	1,146	1,383	1,339	0,342	0,140
	MALE	1,959	1,009	1,202	1,206	1,094	1,304	1,296	0,341	0,139
	FEMALE	1,820	0.996	1,107	1,065	1,038	1,279	1,262	0,326	0,133
	WILDTYPE	1,863	1,000	1,152	0.966	1,031	1,252	1,260	0,352	0,144
	SOD1	1,923	1,009	1,168	1,224	1,093	1,344	1,294	0,329	0,134
7days	**GENERAL**	0,381	0,337	**0,324**	**0,334**	0,352	0,427	0,359	0,039	0,016
	EDL	0,420	**0,320**	**0,285**	0,325	0,345	0,420	0,353	0,056	0,023
	SOLEUS	**0,340**	0,348	0,360	0,341	**0,300**	0,427	0,353	0,042	0,017
	MALE	0,401	0,378	**0,315**	0,356	**0,304**	0,378	0,355	0,038	0,016
	FEMALE	0,353	**0,268**	0,298	**0,250**	0,367	0,416	0,325	0,064	0,026
	WILDTYPE	0,392	0,385	**0,341**	**0,363**	0,382	0,489	0,392	0,051	0,021
	SOD1	0,381	**0,287**	**0,294**	0,298	0,290	0,368	0,320	0,043	0,017
60days	**GENERAL**	**0,766**	**0,724**	0,862	**0,772**	0,826	**0,784**	0,789	0,049	0,020
	EDL	**0,661**	**0,613**	0,739	0,723	0,674	0,693	0,684	0,045	0,019
	SOLEUS	**0,800**	**0,777**	0,888	0,810	0,937	0,825	0,840	0,061	0,025
	MALE	0,839	**0,758**	0,979	**0,829**	0,889	0,873	0,861	0,073	0,030
	FEMALE	**0,664**	0,680	0,741	0,699	0,742	**0,651**	0,696	0,039	0,016
	WILDTYPE	**0,933**	**0,882**	1,043	0,937	1,006	0,941	0,957	0,058	0,024
	SOD1	0,524	**0,469**	0,611	**0,467**	0,540	0,558	0,528	0,055	0,022
90days	**GENERAL**	0,888	**0,751**	0,816	**0,699**	**0,785**	**0,753**	0,782	0,065	0,027
	EDL	0,766	0,690	**0,651**	0,772	**0,682**	0,713	0,712	0,048	0,020
	SOLEUS	0,796	**0,571**	0,780	**0,546**	0,819	0,580	0,682	0,129	0,052
	MALE	0,823	**0,658**	0,733	**0,648**	0,870	0,677	0,735	0,093	0,038
	FEMALE	0,843	0,771	0,840	**0,692**	**0,684**	0,792	0,770	0,070	0,028
	WILDTYPE	0,784	0,722	0,721	**0,686**	**0,707**	0,720	0,723	0,033	0,013
	SOD1	0,986	**0,790**	0,914	**0,726**	0,862	0,803	0,847	0,094	0,038
120days	**GENERAL**	**0,538**	**0,531**	0,781	0,888	0,566	0,613	0,653	0,148	0,060
	EDL	**0,456**	**0,431**	0,845	0,641	0,518	0,490	0,564	0,156	0,064
	SOLEUS	**0,583**	0,611	0,725	1,085	**0,603**	0,709	0,719	0,189	0,077
	MALE	**0,397**	**0,380**	0,550	1,092	0,449	0,467	0,556	0,269	0,110
	FEMALE	**0,563**	**0,552**	0,618	1,092	0,610	0,568	0,667	0,210	0,086
	WILDTYPE	0,366	**0,300**	0,628	0,398	**0,347**	0,376	0,403	0,115	0,047
	SOD1-G93A	**0,625**	**0,636**	0,892	1,162	0,764	0,735	0,802	0,201	0,082
INTRA-AGE								0,622	0,194	0,040
TOTAL								1,292	0,042	0,016

Supplementary material File 3. GeNorm M values of the housekeeping genes.
The individual expression stability values (M)[a], average[b], SD (standard deviation)[c] and SEM (standard error of the mean)[d] of six housekeeping genes. The recommended housekeeping genes to calculate accurate normalization factors in each case are in bold.

	Housekeeping genes								
Group	**Rn18s**	**Gapdh**	**Actb**	**Hprt1**	**Sdha**	**B2m**	**M average**	**SD**	**SEM**
GENERAL	0.401	**0.342**	0.350	**0.341**	0.362	0.363	0.360	0.022	0.009
EDL	**0.327**	0.338	0.371	**0.299**	0.353	0.363	0.342	0.026	0.011
SOLEUS	0.458	**0.332**	**0.328**	0.373	0.369	0.355	0.369	0.047	0.019
MALE	**0.245**	**0.263**	0.307	0.296	0.299	0.281	0.282	0.024	0.010
FEMALE	0.382	**0.305**	0.349	**0.337**	0.367	0.359	0.350	0.027	0.011
NOT DENERVATED	0.431	**0.349**	0.394	0.362	0.409	**0.353**	0.383	0.033	0.014
DENERVATED	0.357	0.320	**0.287**	0.313	**0.288**	0.339	0.317	0.028	0.011
TOTAL							0.343	0.034	0.013

Supplementary material File 4. GeNorm M values of the housekeeping genes in the denervated and control groups.
The individual expression stability values (M) [a], average[b], SD (standard deviation)[c] and SEM (standard error of the mean)[d] of six housekeeping genes in the denervated and control groups. The recommended housekeeping genes to calculate accurate normalization factors in each case are in bold.

3.5. Neurodegenerative Diseases, 2011

Altered *in vitro* proliferation of mouse SOD1-G93A skeletal muscle satellite cells.

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Neurodegener Dis. En revisión.

ALTERED *IN VITRO* PROLIFERATION OF MOUSE SOD1-G93A SKELETAL MUSCLE SATELLITE CELLS

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Short title : Myoblast proliferation in ALS model

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Keywords: amyotrophic lateral sclerosis, skeletal muscle, neurodegenerative disease, SOD1 mutants, satellite cell, proliferation, cell culture.

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 of neuromuscular junctions, and we have previously demonstrated alterations in myogenic regulatory factor (MRF) expression *in vivo* and in activation of myofiber-associated skeletal muscle satellite cells (SMSCs) in mouse model 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 gene expression in SMSC cultures derived from fast-twitch EDL and slow-twitch soleus (SOL) 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-twitch 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. Conclusions: Our data suggest that SMSCs function is impaired in SOD1-G93A satellite cells from the earliest stages of the disease when no critical motor neuron loss has been described.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal neurodegenerative disease. The main hallmark of ALS is 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 hyper-reflexia, 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 (sALS), 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) develops motor neuron pathology similar to that observed in human fALS [3-5]. As mSOD1 can induce motor neuron apoptosis, it was hypothesized that the expression of mSOD1 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 mutant SOD1, 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 and lipid metabolism, altered mitochondrial uncoupling and respiration, take place before the first motor neuron degeneration signs [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 (Igf1) specifically in skeletal muscle delays the disease in mSOD1 mice [16] and it was proposed that the effect of Igf1 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 although retain the ability to become activated,

proliferate and differentiate upon distinct stimuli such as acute injury, exercise or muscle denervation [18]. They further elongate to form new myotubes that fuse each other or with the parental myofiber, to regenerate muscle [18,19]. The paired-box transcription factor *Pax7*, one of the most widely accepted SMSCs markers [20-23], has been shown to be expressed in quiescent and proliferating SMSCs [24-26]. When activated, SMSCs re-enter the cell cycle, co-express the myogenic regulatory factor (MRF) *Myod1* (myogenic differentiation 1) and perform several rounds of proliferation before up-regulating another MRF, myogenin, and subsequently *Mrf4* (myogenic regulatory factor 4) [18,19]. These MRF are muscle-specific members of the basic helix-loop-helix (bHLH) transcription factor superfamily [27] that heterodimerize with ubiquitously expressed 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 MHC isoform expression in satellite cell cultures established from ALS patient derived muscle biopsies [30]. The authors concluded that the ability to differentiate of ALS patients SMSCs is severely impaired. Consistently, evidence recently published by us also suggest 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* and MRF gene expression in satellite cell cultures from fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (SOL) muscles 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 in all stages except in 90 day-old SMSCs cultures where an increment existed. *Pax7* and MRF expression were also modified accordingly to proliferative potential except in latest phases of the disease when a deregulation of the myogenic program 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 12h light/dark cycle at 21–23°C with relative

humidity of 55 %. Food and water were available ad libitum. Animals were sacrified by cervical dislocation.

Satellite cell extraction and culture

Three male B6SJL and SOD1-G93A mice per age group were sacrificed at early presymptomatic (p40), late presymptomatic (p60), symptomatic (p90) and terminal stage (p120). EDL (fast-twitch) and soleus (slow-twitch) muscles were collected and processed in parallel. Muscle satellite cell extraction and culture were carried out following the protocol described by Montarras et al. (Montarras et al 2005). 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 minutes at 37^{o}C. The enzymatic reactions were stopped with fetal bovine serum (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 μm diameter sterile strainer and centrifuged 1800 rpm for 15 minutes at 4^{o}C. Cell pellet was resuspended to 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 possibility that mSOD1 SMSCs display altered susceptibility to freezing or trypsinization. For proliferation and gene expression analysis, total 1000 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 % Ultroser G (Pall-Biosepra). Cells were left to adhere and start proliferating for 3.5 days at 37^{o}C and 5 % CO2. At least four replicate wells were plated for each group of sex, 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 hours till 7.5 days, cell proliferation plate was fixed with 10 % neutral buffered formalin solution (Sigma-Aldrich) for 15 minutes and nuclei were stained with Hoechst 33342 (Bis Benzimide H 33342 trihydrochloride, Sigma-Aldrich) for 5 minutes. After fixation, cells were washed with 1X PBS and five random fields per well were photographed under an

epi-fluorescence microscope (Nikon TE2000-E) at 20X 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 MRF expression

Cells for RNA extraction and cDNA synthesis were washed with cold 1X PBS and transferred to -80^{o}C to wait extraction. The plates were processed according to the Cells-to-cDNA kit´s (Ambion) instructions. Plates were thawed on ice and cells were lysed by adding 100 μl of cell lysis solution. Briefly in order to release RNA from cells and eliminate endogenous RNase activity lysed cells were subjected to heat treatment for 15min at 75°C. Traces of genomic DNA were eliminated with Dnase treatment at 37°C for 15min, 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 μl of dNTPs, 1 μl of random hexamers and 5 μl of RNA and was incubated at 75°C for 3 min. Subsequently, 1 μl 10X reverse transcription buffer, 0.5 μl M-MLV retrotranscriptase and 0.5 μl RNase inhibitor were added and the reaction was incubated at 42°C for 60min followed by 95°C for 10min. For QPCR, cDNA was diluted 1:10 in dH2O and two microliters were used as a template for each reaction (3 replicates per cDNA sample) containing 2.5 μl Fast 2X TaqMan master mix (No AmpErase UNG) and 0.5 μl gene-specific TaqMan assays (Applied Biosystems) for *Pax7* (Mm00834079_m1), *Myod*1 (mM00440387_m1) or myogenin (Mm00446194_m1) in a final volume of 5 μl. Reactions were run using StepOne Plus Real-Time PCR System (Applied Biosystems) using the following conditions; 95^{o}C for 10 minutes and 47 cycles of 95^{o}C for 15 seconds and 60^{o}C for 30 seconds. In each cDNA sample, three endogenous reference genes were amplificated 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 [33-35] 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 [36]. For all primer/probe set 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.

Immunocytochemistry

One well per group was fixed and immunocytochemistry 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 4 % (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 50mM NH4Cl for 10 minutes and saturation for 10 minutes 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 hours at room temperature and visualized with Alexa 546-conjugated anti-rabbit secondary antibody 1:300 (Invitrogen). Nuclei were stained with 1000 ng/ml Hoechst 33342 (Sigma-Aldrich) and mounted with DakoCytomation Faramount fluorescent mounting medium. All cultures used in this study showed a MYOD positive content 70-80 %.

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 (SEM) 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 (Statistic 5.0). Statistical differences were considered significant at $p<0.05$.

Results

To investigate the in vitro proliferation capacity of SMSCs derived from SOD1-G93A mice, we extracted satellite cells from typical fast-twitch EDL and slow-twitch SOL muscles at early presymptomatic (p40), late presymptomatic (p60), symptomatic (p90) and terminal stage (p120) (see materials and methods for details). Harvested cells from each age and muscle group were seeded at standard density and cultured immediately after extraction to avoid potential modification or selection resulting from cryopreservation or trypsinization. Cells were left to adhere and to initiate proliferation for 3.5 days. From 3.5 days to 7.5 days of culture, cells were fixed daily and nuclei were stained with Hoechst and counted. The growth medium used supported both proliferations and differentiation. Myotube formation was recorded (Figure 1A) at 3.5 days (no myotube formation), 7.5 days (small myotubes evident) and 10 days of culture (fully developed myotubes) to determine the myogenic origin of the cells. Additionally, immunostaining for MYOD at 4.5 days revealed that all cultures used in the experiments showed 70-80 % MYOD positive cells (Figure 1B). Despite a slight tendency at advanced culture days, no clear differences were observed between wild-type and SOD1-G93A in the case of EDL-derived SMSCs cultures at p40 (Figure 2A). By contrast, SOL-derived SMSCs of SOD1-G93A mice at p40 possessed lower in vitro proliferation potential than their wild-type littermates (Figure 2B). At late presymptomatic day p60, SOD1-G93A cells proliferated slower than wild-type-derived SMSCs cultures in both muscles under study (Figure 2C and 2D). 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 (Figures 2E and 2F). 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 (Figure 2G and 2H). Because the proliferative capacity of SOD1-G93A SMSCs was increased at p90, at the onset of symptomatic state, we considered the possibility that severe denervation at this stage may temporarily "induce" SMSCs proliferation. To test this hypothesis, we analysed the SMSCs derived from surgically denervated wild-type mice. SMSCs derived from EDL and SOL muscles of denervated limb displayed a superior proliferation rate compared to the SMSCs derived from the unoperated limb (Figure 2I and 2J). This supports the view that gross denervation at the onset of the diasease in SOD1-G93A mice may have stimulatory effect of 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 symptomatic stage the proliferation rate is increased in mSOD1 SMSCs, a result that parallels with 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 analysed the expression of myogenic regulatory factors implicated into the satellite cell determination and myogenic program. *Pax7* and *Myod1* genes were analysed as markers for satellite cell determination and activation, respectively, at 5.5 days of culture when proliferation was prominent without significant differentiation [23,37]. On the other hand, myogenin mRNA, a marker of differentiation process, was measured at day 7.5 when clear myotube formation was observed.

At early presymptomatic day p40, SOD1-G93A SMSCs derived from EDL muscles presented a diminution in the levels of *Pax7* mRNA (Figure 3A, $p<0.05$) compared to wild-type cells. This result was correlated with decreased *Myod1* (Figure 3C, $p<0.05$) and myogenin mRNA levels (Figure 3E, $p<0.01$). In SOL-derived SMSCs, similar tendency to diminution in the levels of *Pax7* mRNA (Figure 3B, $p<0.1$) and *Myod1* (Figure 3D, $p<0.1$), and accompanied with significantly reduced myogenin mRNA levels (Figure 3F, $p<0.05$). Qualitatively similar results were obtained at late-presymptomatic stage p60. Although *Pax7* diminution was only suggestive (Figure 3A, $p<0.1$), levels of *Myod1* and myogenin remained reduced in EDL SMSCs (Figure 3C, $p<0.01$ and Figure 3E, $p<0.01$ respectively). In SOL cultures, *Pax7* mRNA was not altered (Figure 3B) and reduction in *Myod1* and myogenin was suggestive but non-significant (Figure 3D and 3F respectively). At the onset of symptoms (p90), when SOD1-G93A SMSCs displayed similar or relatively increased proliferation (Figures 2E and 2F), similar levels of *Myod1* mRNA were observed in mSOD1 SMSCs compared to the control in EDL and a tendency to diminution in SOL cultures (Figure 3C and 3D). Also myogenin expression reached wild-type levels in EDL (Figure 3E) and even showed tendency to increment in SOL (Figure 3F, $p<0.1$) cultures. This increase in activation and differentiations markers was accompanied with decreased SMSCs quiescence marker *Pax7* (Figure 2B, $p<0.01$). Finally,

at terminal stage (p120), a suggestive increment of *Pax7* transcripts was observed in EDL derived mSOD1 cells (Figure 3A, $p<0.1$); however, there were no differences in *Myod1* levels (Figure 3C) and a tendency to diminution of myogenin mRNA (Figure 3E, $p<0.1$). In SOL cultures, changes were in the same direction as in EDL except for *Myod1* that showed a significant decrease in mSOD1 cells (Figure3D, $p<0.01$). EDL-derived SMSC cultures from denervated limbs expressed more of the three factors analysed, *Pax7*, *Myod1* and myogenin (Figure 3A, $p<0.01$; Figure 3C, $p<0.05$ and Figure 3E, $p<0.01$ respectively). On the other hand, SMSCs from denervated SOL cultures showed a tendency to decrease for *Pax7* (Figure 3B) and *Myod1* transcripts (Figure 3D, $p<0.05$). To summarize, SMSCs obtained from EDL and SOL muscles of SOD1-G93A mice cultured *in vitro* presented diminished levels of *Pax7*, *Myod1* and myogenin mRNA at 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* remained in relatively lower level. Finally, at 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 mutant SOD1 proteins [38], loss of functional motor units [39,40] and reduction in the muscle contractile force [41]. 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 [42]. 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 [39,40,43]. 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 along the disease arises from signals from the cellular niche or represent a cell-autonomous effect of mSOD1 to SMSCs function remains obscure.

To this end, we investigated here the SMSCs proliferation and MRF expression (*Pax7*, *Myod1* and myogenin) in SMSCs cultures established from SOD1-G93A and wild-type mice from 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 approximately half and half of fast glycolytic (type IIb) and fast oxidative-glycolytic (type IIa) fibers, as well as typical slow-twitch SOL containing approximately 60 % slow oxidative (type I) fibers and 40 % type IIa fibers [44]. 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. Consistently, diminished levels of myogenic regulatory factors were found, although sample variability prevented differences of being significant, especially in SOL-derived cultures. Our results are in agreement with earlier report of limited myogenicity of satellite cell cultures obtained from deltoid muscle biopsies in seven ALS patients [30]. In our study, onset of 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 in both muscles and myogenin a shifted towards increment in SOD1-G93A cultures, especially in SOL-derived cultures. By this stage, a critical loss of motor units and tetanic force has been described in both EDL and SOL muscles [13,40,45,46]. Proliferation and turnover of SMSCs at high rate for at least one month after denervation has been also reported [47,48]. 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 terminal stage (p120), a diminished proliferation capacity was observed for SOD1-G93A SMSCs of fast- and slow-twitch muscles. At molecular level, *Pax7* and *Myod1* showed tendency for increased mRNA expression, especially in EDL, possibly reflecting the grave denervation at this stage. However, myogenin displayed marked tendency to be reduced in satellite cell cultures at this stage in agreement with the abortive myogenesis described in ALS patient-derived cells [30] and in denervated skeletal muscle [49]. 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 de-regulation of cell cycle [50]. Additionally, mSOD1 aggregates may inhibit proteasomal machinery and dysregulate 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 [51-53]. Muscle type susceptibility to mSOD1 toxicity varies [39,40,54], and distinct regenerative response has been shown in fast- and slow-twitch myofibers from electrically stimulated [55], denervated and tetrodotoxin-treated muscles [56] and in SOD1-G93A mice [32]. One could, therefore, expect that myogenic cell cultures obtained from fast- and slow-twitch myofibers of SOD1-G93A mice present differences at functional and molecular level. However, compared to the age-matched controls, mSOD1 SMSCs obtained from both fast- and slow-twitch muscles displayed similar, although pathological state-dependent pattern of proliferation and MRF expression. Because mSOD1 in the used model is expressed at similar levels in slow- and fast-twitch muscles [32,57], muscle type susceptibility to mSOD1 toxicity is probably directly linked to the distinct characteristics of motor neurons innervating type I and type II myofibers.
To conclude, our data is consistent with the view that SMSCs 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 reverse, 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 SMSCss 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.

Acknowledgements

We apologise for those authors whose relevant work was not cited due to size restrictions. We thank María Royo and Mamen Carreras (Microscopy and Image Service) for technical assistance with fluorescence microscope, and the I+CS (Aragon Health Sciences Institute) for access to the microscope, and Juan Antonio Castillo for his valuable logistic support. The study was funded by grants Caja Navarra: "Tú eliges, tu decides"; PI071133 and PI10/0178 from the Fondo de Investigación Sanitaria of Spain and PAMER from the Instituto Aragonés de Ciencias de la Salud (PIPAMER 09/09).

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Figure Legends

Figure 1 Proliferation and differentiation process of mouse myogenic cell cultures.

(A) Photographs show the in vitro appearance of satellite cells cultures 3.5, 7.5 and 10 days postplating. (B) The myogenicity of the cells was assessed 4.5 days postplating by co-staining for Hoechst and immunocytochemistry for MYOD. MYOD-positive cells at this stage represented 70-80 % of the population.

Figure 2 Growth curves from wildtype and SOD1-G93A mouse satellite cell cultures.

Pairwise comparison of the satellite cell cultures proliferation curves obtained from SOD1-G93A and wildtype muscles at postnatal ages of 40 days (p40) EDL (A) and SOL (B), 60 days (p60) EDL (C) and SOL (D), 90 days (p90) EDL (E) and SOL (F) and 120 days (p120) EDL (G) and SOL (H); and from denervated EDL (I) and SOL muscles (J). Asterisks denote a student t-test p value <0.05 (*) and <0.01 (**).

Figure 3 Pax7, Myod1 and myogenin expression in satellite cell cultures.

Pax7 (A and B), *Myod1* (C and D) and myogenin (E and F) levels in proliferating and differentiating satellite cells from EDL (A, C, E) and SOL (B, D, F) muscles. In each panel, relative expression in cells from SOD1-G93A (light grey bars) and age-matched wildtype mice (black bars) at postnatal ages of 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 days (A and B) and 7.5 days (C and D) post plating. Asterisks denote a student t-test p value <0.05 (*), p <0.01 (**) and p<0.1 (↓).

Figures

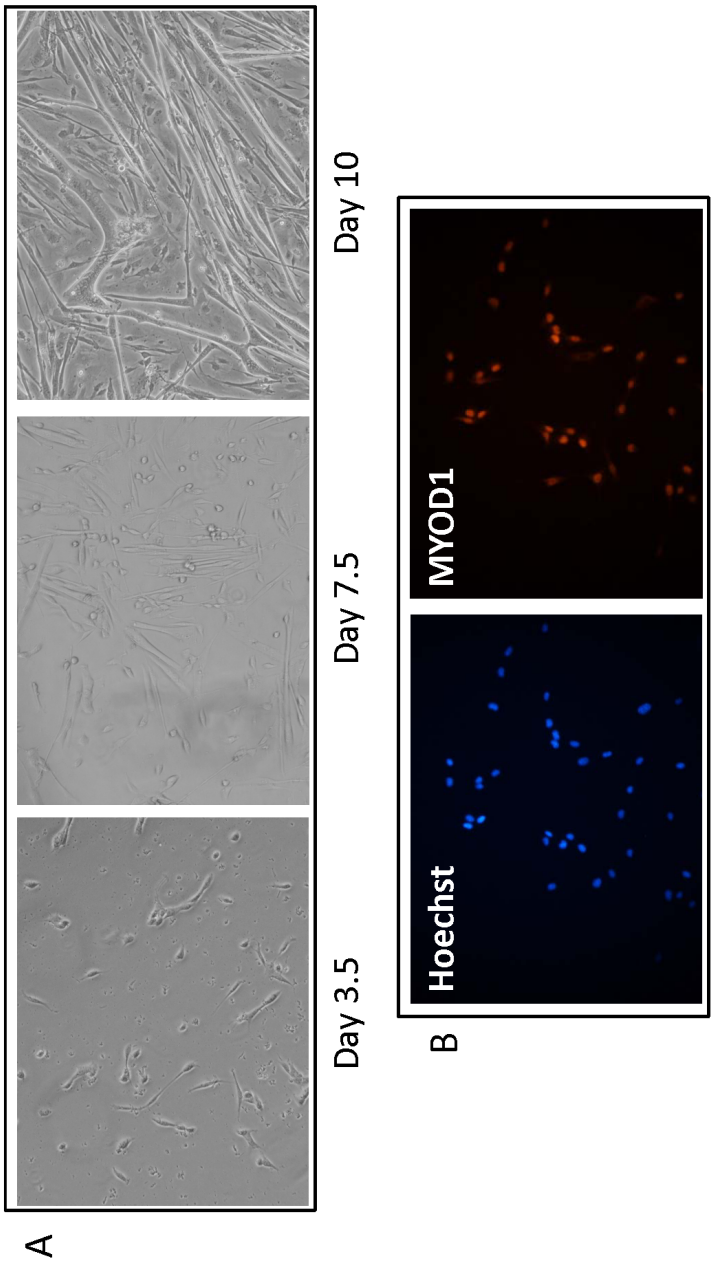
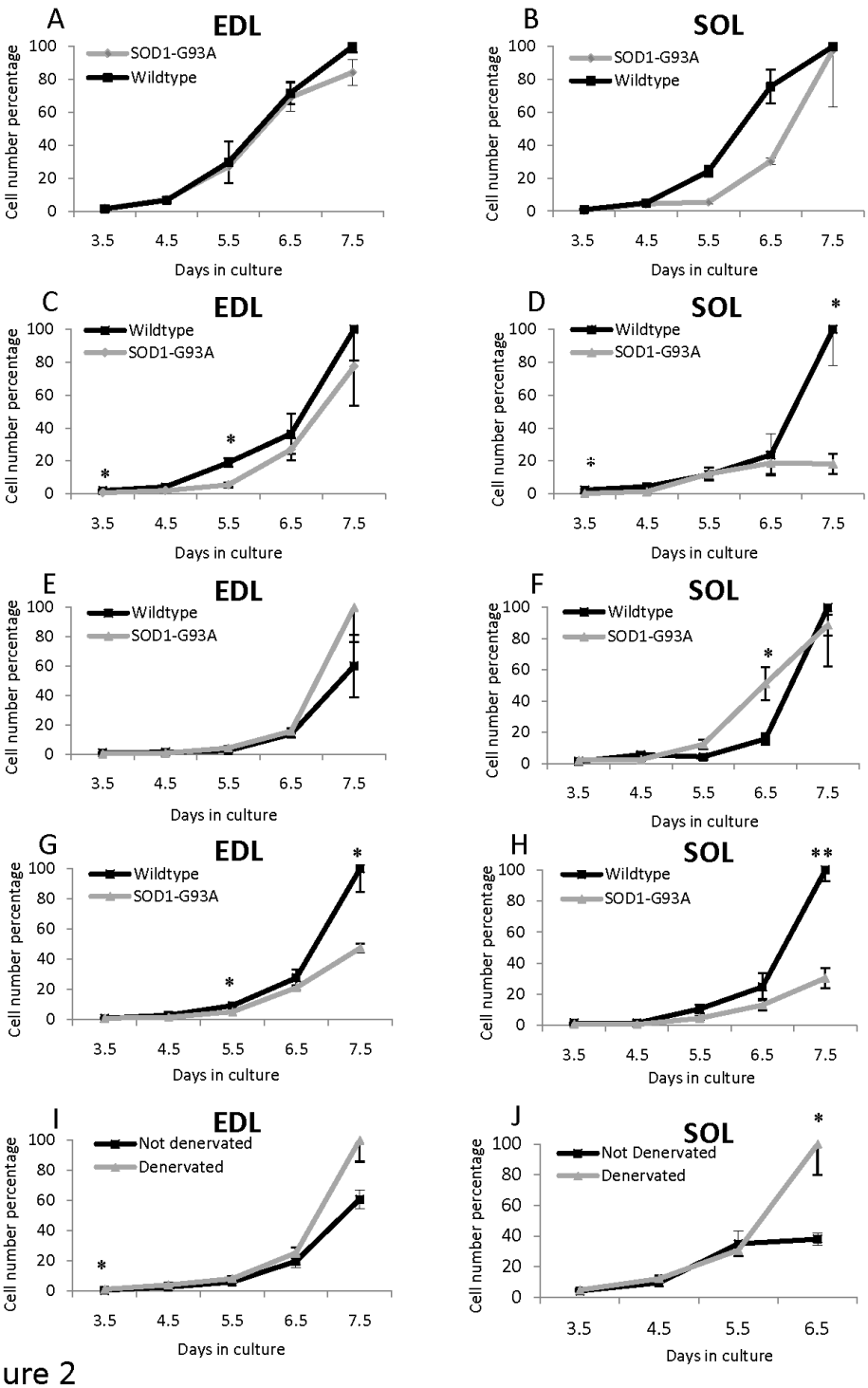


Figure 1.


A
EDL
SOD1-G93A
Wildtype
Cell number percentage
Days in culture
B
SOL
SOD1-G93A
Wildtype
Cell number percentage
Days in culture
C
EDL
Wildtype
SOD1-G93A
Cell number percentage
Days in culture
D
SOL
Wildtype
SOD1-G93A
Cell number percentage
Days in culture
E
EDL
Wildtype
SOD1-G93A
Cell number percentage
Days in culture
F
SOL
Wildtype
SOD1-G93A
Cell number percentage
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G
EDL
Wildtype
SOD1-G93A
Cell number percentage
Days in culture
H
SOL
Wildtype
SOD1-G93A
Cell number percentage
Days in culture
I
EDL
Not denervated
Denervated
Cell number percentage
Days in culture
J
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Not Denervated
Denervated
Cell number percentage
Days in culture

Figure 2

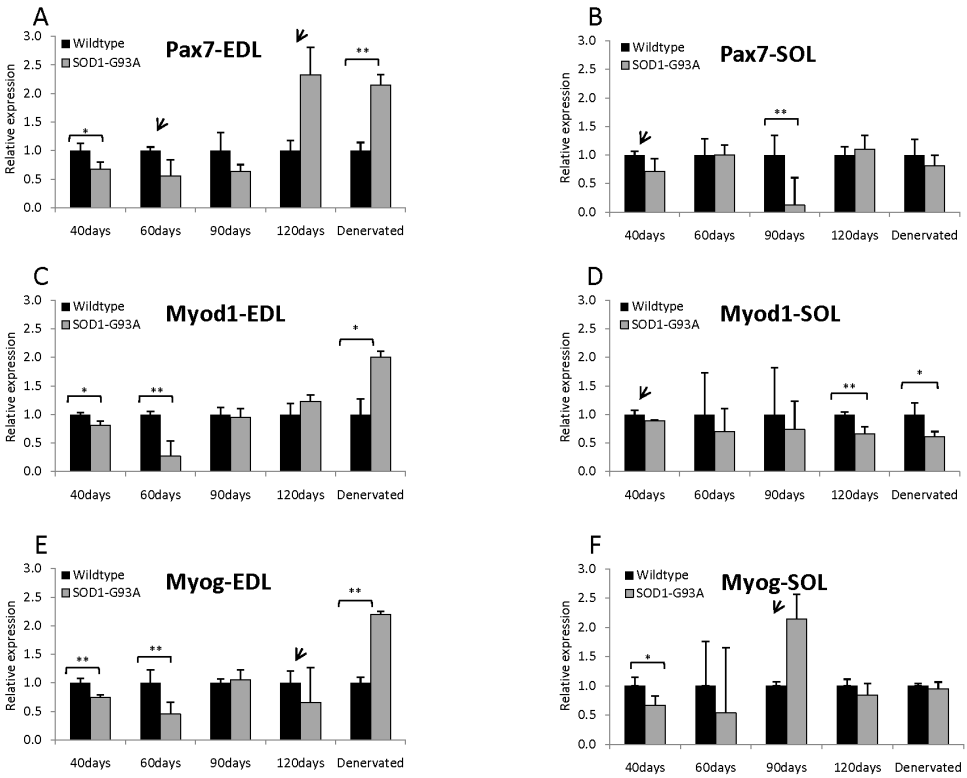
A
Pax7-EDL
B
Pax7-SOL
C
Myod1-EDL
D
Myod1-SOL
E
Myog-EDL
F
Myog-SOL
Wildtype
SOD1-G93A
Relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
40days
60days
90days
120days
Denervated

Figure 3

3.6. Journal of Cellular and Molecular Medicine, 2011

Early myoblast impairment *in vitro* in SOD1-G93A model of Amyotrophic Lateral Sclerosis.

Manzano R, Toivonen JM, Calvo AC, Oliván S, Zaragoza P, Muñoz MJ, Montarras D, Osta R.

J Cell Mol Med. En revisión.

EARLY MYOBLAST IMPAIRMENT *IN VITRO* IN SOD1-G93A MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Abstract and Keywords

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease which leads to the loss of motor units, progressive muscle atrophy and paralysis. Muscle abnormalities have been suggested to precede the motor neuron loss rather than resulting from it and to destabilize neuromuscular junctions resulting in retrograde axonal death ("dying back"). Modified proliferation, myogenic capacity and variations in the quantity and activation status of myofiber-associated skeletal muscle satellite cells (SMSCs) in mouse models and patients of ALS have been reported. However, it is unknown in SMSCs in ALS mice are already affected at very young age. To elucidate whether SMSC impairment is a congenital defect caused by mutant SOD1 (mSOD1) expression we measured *in vitro* proliferation potential and *Pax7* and myogenic regulatory factor (MRF) expression in SMSC cultures derived from fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (SOL) muscles of 1 week old male and female SOD1-G93A mice. SMSCs from young SOD1-G93A mice demonstrated generally lower proliferation potential compared with age-matched controls in both sexes and muscle origins. In male mice, this was accompanied with a reduction in the mRNA and protein levels of *Pax7* and MRFs. In females, induced MRF expression was observed at molecular level in mSOD1 SMSC cultures, although this was not translated into increased proliferation rate. The data supports the view that proliferative capacity is altered in mSOD1 SMSCs from very early postnatal stage where denervation has not yet commenced.

Keywords

ALS, satellite cell, proliferation, muscle, myofiber, cell culture, neurodegeneration, newborn, sex.

Introduction

Amyotrophic lateral sclerosis is a fatal adult onset neurodegenerative disease characterized by the selective and progressive death of both upper and lower motor neurons, leading to a progressive paralysis, respiratory depression and death usually within 2–5 years after onset[1]. Most cases (90%) are classified as sporadic ALS (sALS), as they are not associated with a documented family history. The remaining 10% are defined as familial ALS cases (fALS), from which 10-15% are caused by missense mutations in the gene that encodes Cu/Zn superoxide dismutase 1 (SOD1)[2]. The animal model that best recapitulates the events of human ALS is hemizygote mouse overexpressing the human SOD1 with the G93A mutation[3]. These animals become symptomatic at 90 days of age with hindlimb weakness, jittering and muscle atrophy, followed by progressive paralysis and terminal semi-paralytic stage around 120 days of age [4]. Although the main hallmark of ALS is a loss of upper and lower motor neurons, recent findings suggest that this pathology is non-cell autonomous. Transgenic mice expressing mutant SOD1 (mSOD1) selectively in neurons [5,6], astrocytes[7] or microglia[8] did not lead to motor neuron degeneration. However, the reduction of mSOD1 levels in those cells [9,10] or the proportion of mutant cells in contact with motor neurons sharply delayed the onset and disease progression [8,11-13]. Cells outside the CNS, including fibroblasts [14,15], lymphocytes [16] and skeletal muscle [17-20], are also affected in ALS. Expression of mSOD1 exclusively in the skeletal muscle resulted in limb weakness and motor deficits and was associated with severe pathology involving oxidative damage, protein nitration and marked neuromuscular junction (NMJ) abnormalities. Skeletal muscle satellite cells (SMSCs) are muscle derived progenitors located in the periphery of the myofiber, between the basal lamina and the sarcolemma. They express paired-box transcription factor *Pax7* as specific marker [21]. In adults, SMSC are generally quiescent but retain the ability to proliferate and differentiate upon stimuli such as injury, denervation or acute exercise [22,23]. In these conditions satellite cells activate and re-enter cell cycle co-expressing *Pax7* and *Myod1*, and perform several rounds of proliferation before down-regulating *Pax7*. They exit cell cycle to differentiate and co-express myogenin to elongate and form new myotubes fusing with each other or with the parental myofibers to regenerate damaged muscle [24-27]. If skeletal muscle is a primary target of mSOD1-mediated toxicity[28,29] and effective muscle-targeted therapies seem to act in part through enhanced SMSC activity [30,31], it is of interest to investigate the regenerative potential of these cells in ALS animal models and pa-

tients. Recently, several studies have addressed this issue. We have earlier reported differences in the number and activation status of SMSCs in myofibers from typical fast-twitch and slow-twitch muscles of SOD1-G93A mice at the pre-symptomatic stages (40 day-old) [32]. We also observed differences between the two types of muscles (slow and fast) in the SMSC response, which is consistent with a fiber-type dependent susceptibility to disease reported by other authors[33-35]. *In vitro*, myogenicity of satellite cells is impaired in cultures from ALS patient biopsies[36], and we have observed a diminished proliferative potential of these cells in cultures obtained from SOD1-G93A mice muscles at different stages of the disease (unpublished results). Overall these results indicate that satellite cell mSOD1 toxicity and impaired regenerative response may be a at least partially satellite cell-autonomous mechanism, and not solely mediated by the muscular niche inputs.

However, it is not known if SMSCs are affected in the earliest postnatal stages in SOD1-G93A mice. In young pups, no denervation should exist since there has been a very short postnatal exposure for mSOD1 toxicity and no described mSOD1 accumulation. In the present study, we addressed this question and tested the proliferative potential and *Pax7* and MRF gene and protein expression in SMSC cultures derived from EDL and SOL muscles of seven day-old male and female SOD1-G93A mice. A diminished proliferative capacity of SOD1-G93A satellite cells was observed in both sexes already at this early age. In male mSOD1 cultures MRFs were down-regulated at mRNA and protein level, whereas in female cells induction of the MRFs was observed, although this was not translated into increased proliferative potential.

Materials and Methods

Transgenic mice with the G93A human SOD1 mutation (B6SJLTg[SOD1-G93A]-1Gur) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Hemizygotes were maintained by breeding SOD1-G93A males with wild type females. The offspring were genotyped by PCR amplification of DNA extracted from the tail tissue, as described in The Jackson Laboratory protocol for genotyping SOD1-G93A transgenic mice [37]. Animals were sexed by ZFX/ZFY PCR amplification followed by Hae III restriction enzyme digestion, as described [38]. All experimental procedures were approved by the Ethics Committee of Universidad de Zaragoza and followed the international and the institutional guidelines for the use of laboratory

animals. Mice were housed under a 12h light: 12h dark cycle in 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

Four male and four female B6SJL and SOD1-G93A mice were sacrificed at postnatal age of 7-10 days (p7). Typically fast-twitch EDL and slow-twitch SOL muscles were harvested and processed in parallel following the desctibrd protocol [39]. Isolated muscles of the same genotype, age and muscle were pooled and minced to a slurry with sterile scissors. Subsequently, samples were digested in an enzyme solution containing 0.1% trypsin and 0.1% collagenase (w/v) (Sigma-Aldrich) for 30 minutes at 37^{o}C. The digestion was terminated by inhibiting the anzyme activity with fetal calf serum. Three to four rounds of digestion were performed until the muscle bulk was completely digested. The cell suspension was filtered through a 70 μm diameter sterile strainer and centrifuged at 1800 rpm for 15 minutes and 4^{o}C. Cell pellet was resuspended to DMEM + GlutaMAX (Gibco) and stained with 0.4% trypan blue (Sigma-Aldrich) for viable cell counting [40]. Cultures were established with freshly isolated passage zero cells to avoid possible differences related to susceptibility of SOD1-G93A SMSCs to freezing and trypsinization. For all experiments, 96-well plates coated with Matrigel basement membrane matrix (Becton Dickinson SA) were used and total 1000 cells per well were seeded. The culture medium was composed of 39% F-12 + GlutaMAX (Gibco), 39% DMEM + GlutaMAX (Gibco), 10% fetal calf serum (Gibco) and 2% Ultroser G (Pall-Biosepra). Cells were left to adhere and start proliferating for 2.5 days at 37^{o}C and 5% CO2. Experiments were performed in parallel for cell proliferation, gene expression analysis and immunocytochemistry.

Cell proliferation assay

Starting 2.5 days after the plating and till 7.5 days, cell proliferation plates were daily fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) and the nuclei were stained with Hoechst 33342 (Bis Benzimide H 33342 trihydrochloride, Sigma-Aldrich) for 5 minutes. For cell counting, wells were washed with 1X PBS and five random fields per well were photographed under an epi-fluorescence microscope (Nikon TE2000-E) at 20X magnification and 325 nm (Figure 1A). For each time

point and group, the number of Hoechst positive nuclei of four replicate wells were counted (total 20 fields). For each day of culture, the data of SOD1-G93A and wild type cultures were compared (see below for the statistics). The data from 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 MRF expression

At 5.5 and 7.5 days post plating, plates for mRNA extraction and cDNA synthesis were stopped and processed according to the Cells-to-cDNA kit´s (Ambion) instructions. Cells were washed with cold 1X PBS and briefly transferred to -80ºC. Two biological replicate wells per group were ice-thawed and treated with 100 μl of cell lysis solution per well to disrupt the cells. In order to release RNA from cells and eliminate endogenous RNase activity, lysed cells were heat treated for 15min at 75°C and traces of genomic DNA were eliminated with Dnase treatment at 37°C for 15min, followed by 5 min of heat inactivation at 75°C. Two cDNA synthesis reactions from each RNA sample were performed including a pool of 2 μl of dNTPs, 1 μl of random hexamers and 5 μl of RNA and were incubated at 75°C for 3 min. Subsequently, 1 μl 10X reverse transcription buffer, 0.5 μl M-MLV retrotranscriptase and 0.5 μl RNase inhibitor were added and the reactions incubated at 42°C for 60min followed by 95°C for 10min. For QPCR, two microliters of 1:10 cDNA in dH2O were used as a template. Each reaction (3 replicates per cDNA sample) contained 2.5 μl Fast 2X TaqMan master mix (No AmpErase UNG) and 0.5 μl gene-specific TaqMan assays (Applied Biosystems) for *Pax7* (Mm00834079_m1), *Myod1* (mM00440387_m1) or myogenin (Mm00446194_m1) in a final volume of 5 μl. Reactions were run on a StepOne Plus Real-Time PCR System (Applied Biosystems) using the following termal cycling conditions: 95ºC for 10 minutes and 47 cycles of 95ºC for 15 seconds and 60ºC for 30 seconds. In each cDNA sample, three endogenous reference genes were amplificated using TaqMan assays: *18S ribosomal RNA* (4352930E), *Gapdh* (4352932E), and *β-actin* (4352933E). The geometric mean of the three housekeeping genes was used to normalize target gene expression results [41-43]. Relative gene expression was determined using the 2-$\Delta\Delta$CT method and the data from the wildtype derived cultures for each group as calibrator [44]. For all primer/probe set reaction efficiencies approached 100%.

Immunocytochemistry

At 5.5 and 7.5 days post plating, four wells per group were fixed and processed for immunocytochemistry in parallel to mRNA plates. Cells were rinsed with PBS and fixed in neutral buffered formalin solution 4% (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 50mM NH4Cl for 10 minutes and saturation for 10 minutes with 0.2% (w/v) gelatine in PBS. Two replicate wells were stained for PAX7 and MYOD1 and two for MYOD1 and MYOG. Primary antibodies PAX7 (DSHB), MYOD1 (sc-304, Santa Cruz Biotechnology) and MYOG (Dako, Clone: F5D) were incubated for 2 hours at room temperature and visualized with Alexa 488 or 546-conjugated anti-mouse or anti-rabbit secondary antibodies (Invitrogen). Nuclei were stained with 1000 ng/ml Hoechst 33342 (Sigma-Aldrich) and mounted with DakoCytomation Faramount fluorescent mounting medium. Finally PAX7, MYOD1 and MYOG positive cells were counted in five random fields per well of the two (PAX7 and MYOG) or four (MYOD1) wells per group (Figure 1B, C and D). Results from each well were averaged and positive SOD1-G93A cells for all proteins in were expressed as relative numbers to each control group.

Statistical analysis

In the proliferation assay, total cells from five photographic fields from every replicate well were manually counted and the mean and standard error of mean (SEM) for each group was calculated from the value of four replicate wells. For the QPCR relative quantification, 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). Finally for immunocytochemistry results, positive cells were counted in five random fields per well and results from each well were averaged for statistical analysis. Data obtained from SOD1-G93A and control groups were compared using Student's t-test (Statistic 5.0). Statistical differences were considered significant at $p<0.05$* and $p<0.01$**. Black arrows indicate $p<0.1$.

Results

To investigate the in vitro proliferation capacity of SMSCs derived from neonatal SOD1-G93A mice in both sex and fiber-type (fast and slow), we extracted satellite cells from typical fast-twitch EDL and slow-twitch SOL muscles of both sexes at

asymptomatic stage (p7). Harvested cells were used immediately after extraction to avoid potentially biased results derived from cryopreservation or trypsinization process. Cells from both sexes and muscle groups were seeded at standard density and left to adhere and to initiate proliferation for 3.5 days. From 3.5 days to 7.5 days of culture, cells were fixed daily and nuclei were stained with Hoechst and counted. The growth medium used supported both proliferation and differentiation (Figure 1E and F). Although all data points did not reach significance SMSC cultures derived from SOD1-G93A mice had lower proliferation capacity compared to their wild type littermates (Figure 2). This was consistently observed in male (Figure 2A-B) and female (Figure 2C-D) derived satellite cells and in both EDL (Figure 2A and 2C) and SOL (Figure 2B and 2D) muscles. To shed light into the molecular mechanisms that may underline the impaired proliferative potential in SOD1-G93A satellite cells, we determined the mRNA and protein levels of *Pax7* and *Myod1*, both factors implicated into the satellite cell determination and activation, respectively, at 5.5 days of culture when proliferation was prominent without significant differentiation (Figure 1E) [45,46]. On the other hand, myogenin, a marker of differentiation process, was measured at day 7.5 when myotube formation was evident (Figure 1F). In SMSC cultures derived from male mice, although this mostly did not always reach significance, we observed a consistent tendency towards decreased *Pax7* and *Myod1* factors in SOD1-G93A cells both in mRNA and protein levels (Figure 3). This result was coincident for EDL (Figure 3A mRNA and 3B protein) and SOL muscles (Figure 3C mRNA and 3D protein). Myogenin was not affected in by mSOD1 expression in cells derived from EDL (Figure 3A mRNA and 3B protein) and showed tendency to diminished levels in those from SOL (Figure 3C, mRNA and 3D, protein $p<0.1$). Again, we observed very similar changes both in mRNA and protein level. On the other hand, although SOD1-G93A female SMSCs also showed a lower proliferation rate compared to their wild type littermates, this was not correlated with lower MRF expression (Figure 4). Lower *Pax7* mRNA transcripts and protein levels were found both in EDL (Figure 4A for mRNA, 4B for protein) and SOL (Figure 4C for mRNA, 4D for protein) derived female cells. However, except in SOL mRNA ($p<0.05$), none reached significance. Tendency towards the increased *Myod1* and myogenin was found both in EDL and SOL cultures. Down regulation of *Pax7*, accompanied by up-regulation of *Myod1* and myogenin has been described to be essential for the correct SMSC activation and differentiation [24,46,47]. Therefore regarding the female results, we suggest that myogenic program is mildly exacer-

bated in SOD1-G93A female SMSC cultures, but this is not accomplished by the increment in the proliferation rate of these cells either in EDL or SOL muscles. In summary our results indicate impaired myogenic program in male SMSCs, associated with a reduction in the proliferative potential in vitro. By contrast, female SOD1-G93A SMSCs presented an induction of the MRFs that was not reflected into higher proliferation rate of the cells.

Discussion

Most studies describe the first appearance of muscle pathology in the mouse model of ALS SOD1-G93A around 40 days of age with reduction in the muscle contractile force [48], aggregation of mSOD1 proteins [49] and loss of functional motor units [33,34]. However, although defects related to mSOD1 toxicity in CNS have been described even during embryonic development (see below), potential alterations in embryonic or early postnatal skeletal muscle are unknown. Alterations *in vitro* in motor neurons derived from mSOD1 mouse embryos have been described in excitability, AMPA glutamate postsynaptic receptor expression, and increased sensibility to cell death through up-regulation of Fas and consequent activation of apoptotic pathway[50-53]. . Extracellular recordings from spinal ventral roots in vitro demonstrated also decreased pharmacologically induced motor activities in one week old mSOD1 (G85R) mice [54]. Finally, overexpression of mutant human SOD1 in zebrafish embryos induces a dose-dependent motor axonopathy [55] and deficits in the retrograde axonal transport have been detected in SOD1-G93A motor neuron cultures as early as 13 days of gestation [56]. All these data support the view that mSOD1 toxicity interferes cellular function from the earliest stages of the development, at least in motor neurons. As accumulating evidence suggests that motor neuron degeneration proceeds in a "dying back" pattern, initiating from the skeletal muscle to the neuromuscular junction and distal axon [57,58]. It is of interest the study if also skeletal muscle is subject to such early mSOD1-induced insults. Muscle fiber-type differences have been described in the time-course and severity of the mSOD1 muscle pathology [33,34,59], and our previous reports have revealed distinct myogenic response in SOD1-G93A fast and slow muscles *in situ* [32]. Hence, we analyzed here the proliferation and *Pax7* and MRF gene and protein expression in satellite cell cultures obtained from typical fast EDL and slow SOL muscles from newborn SOD1-G93A mice and wild type littermates. Also gender-

dependent heterogeneity has been reported in the progression and lifespan of the SOD1-G93A mouse model of ALS [4]. We studied both males and females to further decipher if the sex-related differences arise from signals at the cellular niche or reflect cell-autonomous differential myogenic response to mSOD1 toxicity from the earliest postnatal life. Diminished proliferative capacity of was observed in mSOD1 SMSCs from one week old mice which is in agreement with our previous results in early and late presymptomatic SOD1-G93A mice myogenic cell cultures (unpublished results) and with those reporting lower myogenic capacity in cultured satellite cells from ALS patients[36]. However, although SMSC cultures from patients were reported to be generally difficult to establish[36], we have found that in SOD1-G93A mice cultures can be routinely established from all ages. In the present study, we also found a suggestive correlation with the reduced proliferation rate of male mutant cells with lower *Pax7* and MRFs levels. Although relative expression of these factors was altered mildly, the result was consistent in both mRNA and protein level. Several factors may contribute to the lower proliferation rate, including interactions between SOD1-G93A with antiapoptotic of cell cycle regulators[36]. Additionally, the multiple rounds of proliferation in the cultured mSOD1 cells may increase ROS production, oxidative species being inhibitors of myogenesis in vitro in embryonic culture [36,60-62]. This could explain generally decreased levels of MRFs in male-derived SMSCs. By contrast, female derived SMSCs showed decreased levels of *Pax7* and increased *Myod1* and myogenin expression (mRNA and protein) which resembles the typical pattern of the regenerative myogenesis [24,46,47]. Gender differences in the MRF expression in SOD1 SMSCs may arise from such heterogeneity in the ROS chemistry buffering. For example, primary neurons from developing female embryos express higher levels of ROS-detoxifying enzyme glucose-6-phosphate dehydrogenase [63] whereas fetal male neurons are more sensitive to ischemia and nitrosative stress due to reduced levels of glutathione, which also regulates ROS levels [64]. Although muscle data remains scarce, it is possible that female mSOD1 cells may be more efficient against the effects of the oxidative stress (such as inhibition of myogenesis), and therefore competent in MRF up-regulation as observed. However, the increase in MRFs is not translated into a higher proliferative response of the SMSCs. The implications for this uncoordinated response may include mSOD1 aggregate-mediated interruption of cell cycle as these have been observed in embryonic skeletal muscle of C. elegans [65] or physical interaction between G93A-SOD1 and cell cycle regulatory proteins such as Bcl-2 and p27 [66]. Additionally, mSOD1

aggregates may inhibit proteasomal machinery and deregulate cellular activities such as protein folding and organelle function (Golgi, endoplasmic reticulum, and mitochondria), all known consequences of ALS. In summary, our results show that *in vitro* proliferative capacity of SOD1-G93A SMSCs from mice of very young postnatal age is reduced. This is true for SMSCs harvested from fast- and slow-twitch muscles, which are differentially affected by denervation in vivo in ALS patients and models. The data supports the view that the fast muscle susceptibility in ALS is mainly due to a conjunction of signals from the cellular environment in vivo and from distinct characteristics of motor neurons innervating the different types of myofibers, and not from differential cell-autonomous effects of mSOD1 in these two muscle fiber types. Finally, sex dimorphism was observed in the mutant SOD1 SMSC myogenic regulatory factor expression. Since hormonal effects cannot be attributable at this early postnatal stage, it is likely that this heterogeneity may reflect intrinsic differences into mSOD1 toxicity response in the two sexes. Further studies are warranted to decipher the cellular mechanisms and signalling pathways contributing to the impaired satellite cell proliferation and to further depict the implications of these results to the etiopathogeny of ALS. In conjunction, we consider our novel results of significant value to guide future research on muscle targeted therapies for ALS.

Acknowledgements

We thank María Royo and Mamen Carreras (Microscopy and Image Service) for technical assistance with fluorescence microscope, and the equipment was donated by the I+CS (Aragon Health Sciences Institute); and Juan Antonio Castillo for his valuable logistic support. The study was supported by grants from Caja Navarra: “Tú eliges, tu decides”; PI071133 and PI10/0178 from the Fondo de Investigación Sanitaria of Spain (to RO) and PAMER from the Instituto Aragonés de Ciencias de la Salud (PIPAMER 09/09)(to JMT, ACC, SO and RM) and RM received a graduate studentship award from the Government of Aragón (Spain) . RM and JMT performed the research; RM, PZ and RO designed the research study and wrote the paper; RM, RO and DM analyzed the data and ACC, SO and MJM contributed essential reagents and tools.

Conflicts of interest statement

"The authors confirm that there are no conflicts of interest".

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Figure Legends

Figure 1 Proliferation and differentiation process of mouse myogenic cell cultures and Hoechst, PAX7, MYOD1 and MYOG staining.

Figure 1 shows the aspect of the satellite cell cultures after Hoechst (A), PAX7 (B), MYOG (C) and MYOD1 staining (D). Pictures E and F show SMSC cultures 5.5 days (no myotubes evident) and 7.5 days post plating respectively (clear myotube formation).

Figure 2 Satellite cell culture proliferation curves.

Figure 2 shows the pairwise comparison of the satellite cell culture proliferation curves obtained from seven days old SOD1-G93A (grey bars) and wild type muscles (black bars)from EDL(A) and SOL(B) male muscles, and EDL (C) and SOL (D)female muscles. Error bars represent mean± standard error of the mean converted to percentage of the maximum value for each comparison (see material and methods for details). Asterisks denote a student t-test p value <0.1 (↓), <0.05 (*) and <0.01 (**).

Figure 3 *Pax7*, *Myod1* and myogenin gene and protein expression in SOD1-G93A and wild type EDL and SOL muscles SMSC cultures from male mice at p7.

Pax7, *Myod1* and myogenin gene (mRNA) (A and C) and protein expression (B and D) in EDL and SOL muscle SMSC cultures from SOD1-G93A (grey bars) and wild type (black bars) male mice. Error bars represent mean± standard error of the mean. Asterisks denote a student t-test p value <0.1 (↓), <0.05 (*) and <0.01 (**).

Figure 4 *Pax7*, *Myod1* and myogenin gene and protein expression in SOD1-G93A and wild type EDL and SOL muscles SMSC cultures from female mice at p7.

Pax7, *Myod1* and myogenin gene (mRNA) (A and C) and protein expression (B and D) in EDL and SOL muscle SMSC cultures from SOD1-G93A (grey bars) and wild type (balck bars) female mice. Error bars represent mean± standard error of the mean. Asterisks denote a student t-test p value <0.1 (↓), <0.05 (*) and <0.01 (**).

Figures

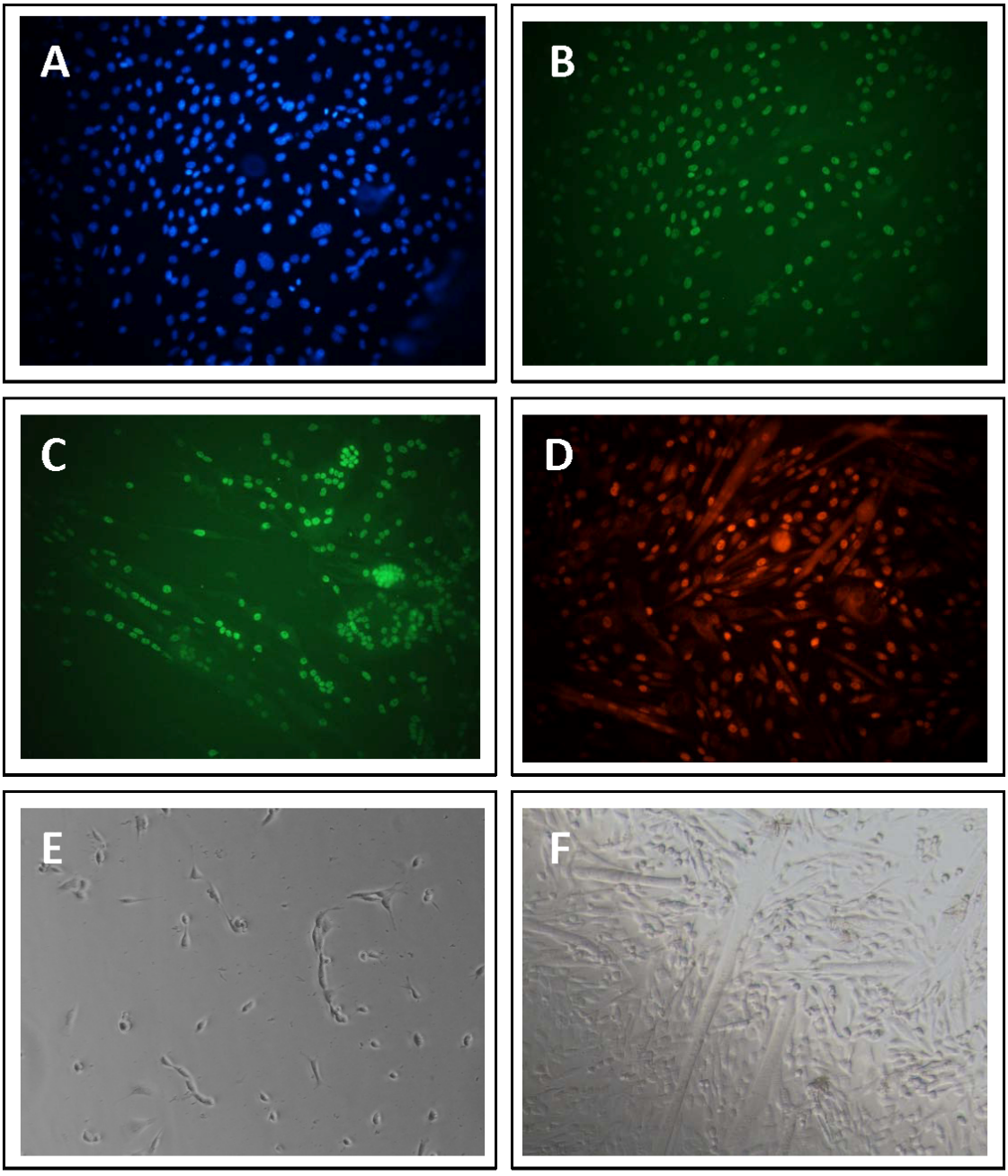


Figure 1.

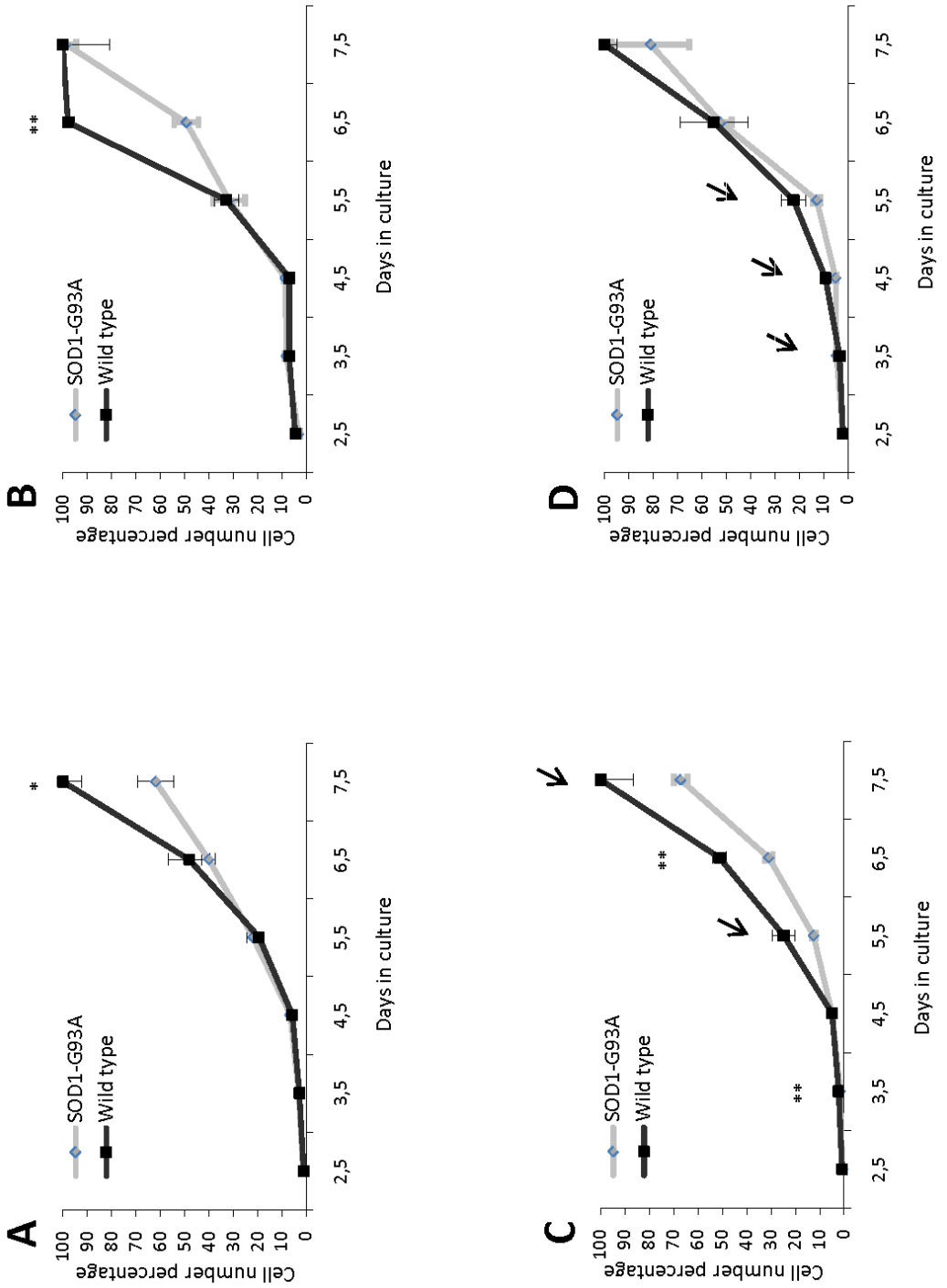

A
B
C
D
Cell number percentage
Days in culture
SOD1-G93A
Wild type
0
10
20
30
40
50
60
70
80
90
100
2,5
3,5
4,5
5,5
6,5
7,5
*
**

Figure 2.

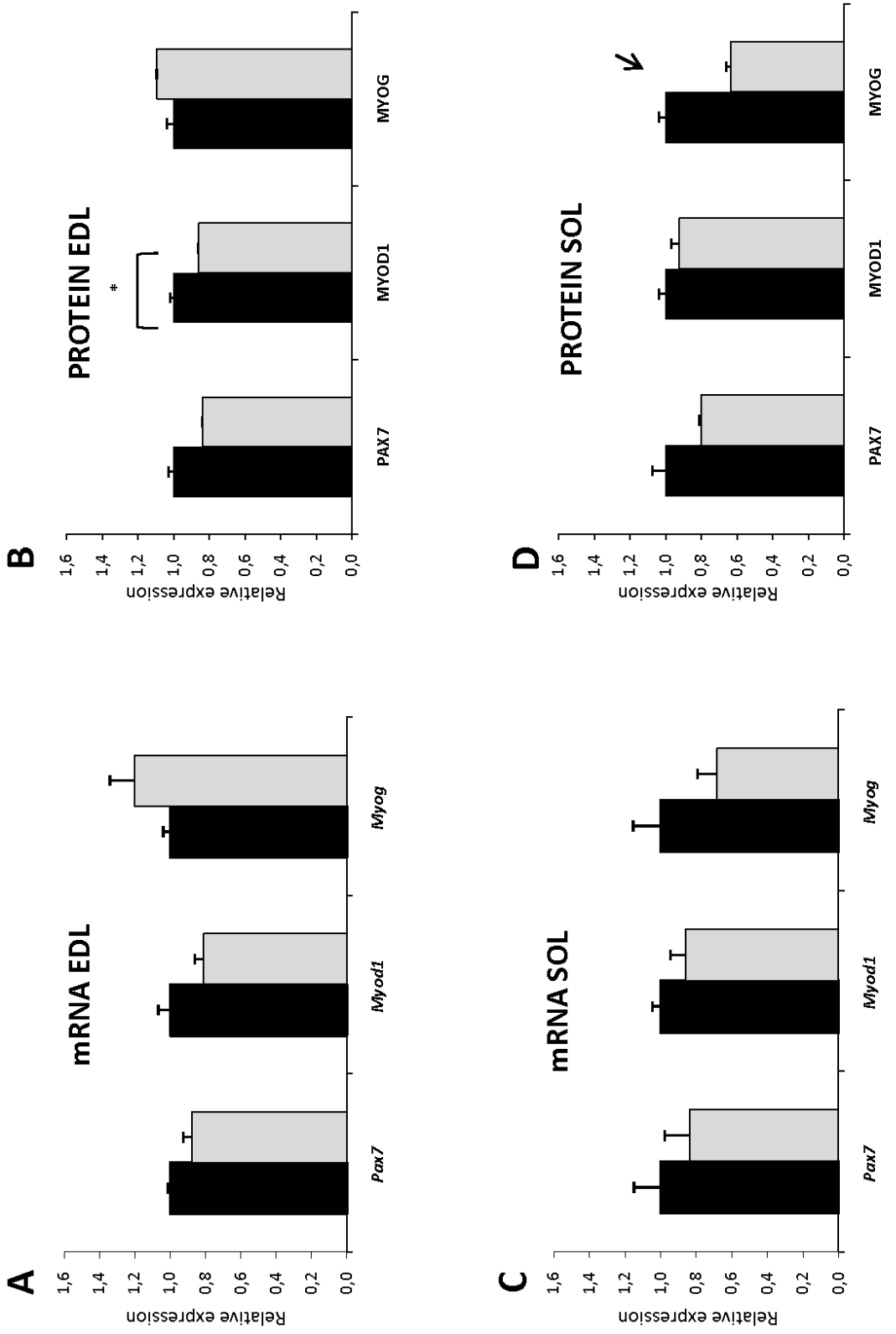

A
mRNA EDL
Relative expression
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Pax7
Myod1
Myog
B
PROTEIN EDL
*
PAX7
MYOD1
MYOG
C
mRNA SOL
D
PROTEIN SOL

Figure 3.

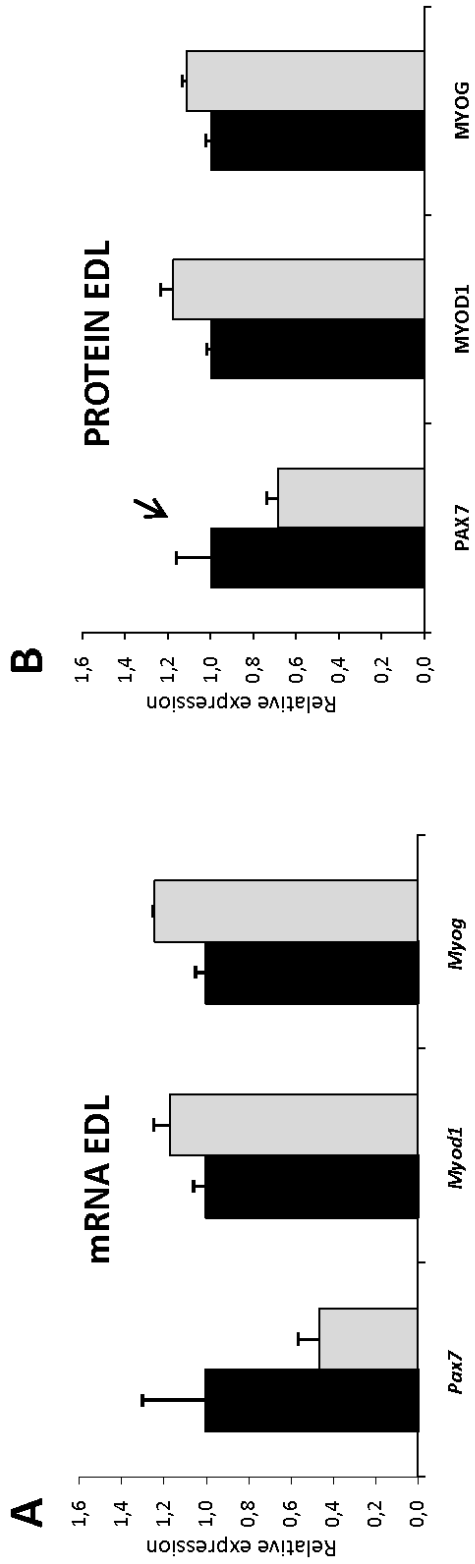
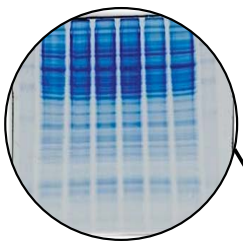

A
mRNA EDL
Relative expression
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Pax7
Myod1
Myog
B
PROTEIN EDL
Relative expression
PAX7
MYOD1
MYOG
C
mRNA SOL
*
Pax7
Myod1
Myog
D
PROTEIN SOL
PAX7
MYOD1
MYOG

Figure 4.



CONCLUSIONES

4. CONCLUSIONES

De los resultados obtenidos a partir de las experiencias realizadas en el presente estudio, de su discusión y contraste con otras aportaciones, pueden deducirse las siguientes conclusiones:

1. Se ha observado una activación de los mecanismos moleculares de respuesta muscular en el modelo animal SOD1-G93A de ELA anterior a la pérdida masiva de inervación. Esta activación evoluciona proporcionalmente a la denervación y pérdida de conexiones neuromusculares. Sin embargo, el incremento de transcritos no se corresponde, especialmente en estadios terminales, con un aumento de la traducción proteínica debido al efecto de la sobre-expresión de la SOD1 humana mutada.

2. En estadios tempranos (40 días) existe un menor número de células satélite musculares en las miofibras del modelo SOD1-G93A *in vivo*. Posteriormente, debido a la influencia del nicho celular, este número varía según la edad de los animales y el tipo de fibra estudiada.

3. La capacidad proliferativa de las células satélite musculares en ambos tipos de fibras ("fast" y "slow") está disminuida en el modelo animal SOD1-G93A excepto en la fase sintomática (90 días) cuando la denervación estimula dicha capacidad al igual que en músculos axotomizados.

4. La alteración del número y comportamiento de las células satélite musculares en el modelo de esclerosis lateral amiotrófica SOD1-G93A desde los estadios neonatales hasta los terminales se presenta como una futura diana terapéutica en esta patología.

CONCLUSIONS

The results obtained from experiments carried out in this study, its discussion and contrast with other contributions, prompted us to the following conclusions :

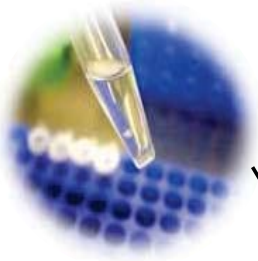
1. The activation of muscular mechanisms of molecular response to injury in the ALS mouse model SOD1-G93A is prior to the massive loss of innervation. This activation evolves proportionally to the denervation and loss of neuromuscular

connections. However, the increment of transcripts does not correlate with an increase in protein translation due to the effect of mutant SOD1 expression.

2. In the early presymptomatic stages of the disease (40 days old) a reduction in the satellite cell number per myofiber has been described in the SOD1-G93A mouse model *in vivo*. Subsequently, due to the cellular niche influence, the number fluctuates depending on the age of the mice and muscle fiber type.

3. The proliferative capacity of satellite cells derived from fast- and slow-twitch myofibers is diminished in the SOD1-G93A mouse model of ALS. The exception comes at symtomatic stage (90 days old) when denervation exacerbates the proliferation ability of these cells as in axotomized mice.

4. The disturbance of the number and behaviour of SOD1-G93A skeletal muscle satellite cells from the earliest neonatal to the terminal stages of the disease represents a future therapeutic target in this pathology.



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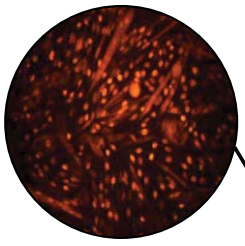

APÉNDICES

6. APÉNDICES

6.1. Apéndice 1.

6.1.1. Características de las revistas

En la tabla se muestran las características de las revistas en las que han sido publicados o enviados los manuscritos que conforman esta Tesis Doctoral. Se especifican los nombres de las revistas así como su índice de impacto (Journal of Citation Reports, Web of Knowledge), el área temática a las que pertenecen y el año y la fase de publicación en la que se encuentran.

Manuscritos	Nombre de la revista	Índice de impacto	Área temática	Año de publicación	Carta de aceptación
Manuscrito 1	Neurodegenerative Diseases	3,791	Clinical Neurology Neurosciences	2011	Publicado
Manuscrito 2	Stem Cell Reviews and Reports	6,774	Cell Biology Medicine, Research and experimental	2011	Adjunto carta aceptación
Manuscrito 3	Journal of Cellular Biochemistry	3,122	Biochemistry and Molecular Biology Cell Biology	2011	Adjunto carta aceptación
Manuscrito 4	Biochemical and Biophysical Research Communications	2,595	Biochemistry and Molecular Biology	2011	Publicado
Manuscrito 5	Neurodegenerative Diseases	3,791	Clinical Neurology Neurosciences	-	En revisión
Manuscrito 6	Journal of Cellular and Molecular Medicine	4,608	Cell Biology Medicine, Research & Experimental	-	En revisión

Cuadro 1: *Características principales de las revistas de publicación.*

6.1.2. Carta de aceptación manuscrito 2

15 April 2011

Dear Rosario Osta:

We are pleased to inform you that your manuscript, "Quantity and activation of myofiber-associated satellite cells in a mouse model of amyotrophic lateral sclerosis" has been accepted for publication in Stem Cell Reviews and Reports. For queries regarding your accepted paper, please contact Jonathan Rapay.
Please remember to always include your manuscript number, #STCR-352R1, whenever inquiring about your manuscript. Thank you.

Best regards,
The Editorial Office
jonathan.rapay@springer.com

6.1.3. Carta de aceptación manuscrito 3

Date:12-May-2011

Ref.:JCB-11-0251

Dear Dr. Osta:

I am pleased to inform you that your manuscript, "Sex, Fiber-Type And Age Dependent In Vitro Proliferation Of Mouse Muscle Satellite Cells", is acceptable for publication in Journal of Cellular Biochemistry.

Effective immediately, papers accepted for publication in JCB will now publish online as Accepted Articles within 5-7 days after acceptance and will be indexed by PubMed within 48 hours of release into the public domain. Papers will be published in final format in print and this version will replace the Accepted Article (unedited) version online. Please note that accepted article publication makes it incumbent upon the authors to submit their revisions with little or no alterations necessary.

Urgent, regarding your proofs: please watch for an email notification to come from JCBprod@wiley.com within four weeks of acceptance. It is critical that this document not be accidentally deleted or screened out as spam.

Proofs must be returned within 48 hours. If you have not received your proofs after four weeks, you may follow up directly with production via email or you may notify us. Confirmation of receipt shall come from JCBprod@wiley.com within 24-48 hours of the return of corrected proofs.

Please feel free to contact our editorial office if you have any questions about this process. For the copyright transfer agreement, or if you are unsure about formatting, please consult the instructions to authors that are given under the "Instructions and Forms" button at the upper left-hand corner of the sign in screen for the Journal of Cellular Biochemistry website. I look forward to seeing more of your work in the future.

Sincerely,

Dr. Gary Stein
Executive Editor

6.2. Apéndice 2. Contribución del doctorando y renuncia de coautores no Doctores

Dña. Rosario Osta Pinzolas, como directora de la presente memoria presentada por Raquel Manzano Martínez para optar al grado de Doctora certifica que,

Todos los coautores de los manuscritos presentados en dicha memoria son Doctores, a excepción de Dña. Sara Oliván García, quien renuncia expresamente a presentar los cuatro manuscritos de los que es coautora como parte de otra Tesis Doctoral para lo que firma además el presente documento.

A continuación se relatan los coautores Doctores de dichas publicaciones:

Janne M. Toivonen
Ana C. Calvo
María Moreno-Igoa
María J. Muñoz
Pilar Zaragoza
Alberto García-Redondo
Rosario Osta
Didier Montarras
Francisco-Javier Miana-Mena
Clementina Rodellar

Firmado: En Zaragoza, a 15 de Junio de 2011

La directora de tesis Dra. Rosario Osta Pinzolas

La única coautora no Doctora Dña. Sara Oliván García