MUSCLE REGENERATION POTENTIAL AND SATELLITE CELL ACTIVATION PROFILE DURING RECOVERY FOLLOWING HINDLIMB IMMOBILIZATION IN MICE

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**MUSCLE REGENERATION POTENTIAL AND SATELLITE CELL
ACTIVATION PROFILE DURING RECOVERY FOLLOWING HINDLIMB
IMMOBILIZATION IN MICE**

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35 **ABSTRACT**

36 Reduced muscle activity leads to muscle atrophy and function loss in patients and
37 animal models. Satellite cells (SCs) are postnatal muscle stem cells that play a pivotal
38 role in skeletal muscle regeneration following injury. The regenerative potential,
39 satellite cell numbers, and markers during recovery following immobilization of the
40 hindlimb for seven days were explored. In mice exposed to seven days of hindlimb
41 immobilization, in those exposed to recovery (seven days, splint removal), and in
42 contralateral control muscles, muscle precursor cells were isolated from all hindlimb
43 muscles (fluorescence-activated cell sorting, FACS), and SCs and muscle regeneration
44 were identified using immunofluorescence (gastrocnemius and soleus) and electron
45 microscopy (EM, gastrocnemius). Expression of *ki67*, *pax7*, *myoD*, and *myogenin* was
46 quantified (RT-PCR) from SC FACS yields. Body and grip strength were determined.
47 Following seven-day hindlimb immobilization, a decline in SCs (FACS,
48 immunofluorescence) was observed together with an upregulation of SC activation
49 markers and signs of muscle regeneration including fusion to existing myofibers (EM).
50 Recovery following hindlimb immobilization was characterized by a program of muscle
51 regeneration events. Hindlimb immobilization induced a decline in SCs together with an
52 upregulation of markers of SC activation, suggesting that fusion to existing myofibers
53 takes place during unloading. Muscle recovery induced a significant rise in muscle
54 precursor cells and regeneration events along with reduced SC activation expression
55 markers and a concomitant rise in terminal muscle differentiation expression. These are
56 novel findings of potential applicability for the treatment of disuse muscle atrophy,
57 which is commonly associated with severe chronic and acute conditions.

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INTRODUCTION

Skeletal muscle dysfunction and mass loss are characteristic features in patients with chronic diseases including cancer of several types and acute conditions such as critical illness. Impaired muscle function and atrophy limit the patients' quality of life as exercise tolerance and daily life activities can be seriously hampered especially following prolonged intensive care unit (ICU) stay. Furthermore, skeletal muscle dysfunction and atrophy have also been shown to predict morbidity and mortality in patients regardless of the underlying condition (Barreiro et al, 2015a; Fearon et al, 2011; Maltais et al, 2014; Marquis et al, 2002; Seymour et al, 2010; Vogelmeier et al, 2017; Barreiro et al, 2015b; Patel et al, 2014; Swallow et al, 2007). Several factors and biological mechanisms have been demonstrated to be involved in the pathophysiology of skeletal muscle mass loss (Barreiro and Sznajder, 2013; Barreiro et al, 2015a; Barreiro and Gea, 2015; Barreiro and Gea, 2016; Barreiro, 2017; Maltais et al, 2014). Deconditioning and reduced muscle activity are counted among the most relevant factors leading to muscle atrophy and loss of function in patients (Barreiro and Gea, 2015; Barreiro et al, 2015a; Fearon et al, 2011; Maltais et al, 2014; Marquis et al, 2002; Seymour et al, 2010) and animal models (Chacon-Cabrera et al, 2016; Chacon-Cabrera et al, 2017a).

Biological mechanisms such as increased oxidative stress, mitochondrial alterations, enhanced protein breakdown, epigenetic events, and signaling pathways mediate muscle mass loss in experimental models of cancer-induced cachexia, chronic heart failure, and disuse muscle atrophy and in cachectic patients with chronic respiratory conditions (Barreiro et al, 2016; Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2016; Chacon-Cabrera et al, 2017b; Chacon-Cabrera et al, 2017a; Fermoselle et al, 2012; Puig-Vilanova et al, 2014b; Puig-Vilanova et al,

2014a;Puig-Vilanova et al, 2015;Barreiro and Sznajder, 2013;Barreiro and Gea, 2015;Barreiro and Gea, 2016). Recently, we have demonstrated that a specific program of molecular and cellular events characterized by increased muscle proteolysis and injury was observed in the gastrocnemius of mice following hindlimb unloading for several time-points, while reloading of the muscle improved all those alterations (Chacon-Cabrera et al, 2016). Additionally, in the same mouse model, muscle-enriched microRNAs, especially miR-486, through **paired box (pax)7** regulation was shown to delay muscle cell differentiation, probably via acetylation of fork-head box (FoxO)1 and 3, in the unloaded gastrocnemius (Chacon-Cabrera et al, 2017a). Interestingly, reloading of the muscle after splint removal induced a rise of the histone deacetylase sirtuin-1 along with a decrease in acetylation levels of the transcription factors FoxO1 and 3, thus enabling the muscle fibers to regenerate in the mice (Chacon-Cabrera et al, 2017a). We concluded that the kinetics of the biological events leading to skeletal muscle mass loss and recovery were tightly regulated in the gastrocnemius of mice exposed to different time-points of muscle unloading and reloading (Chacon-Cabrera et al, 2016;Chacon-Cabrera et al, 2017a). Whether the potential of muscle regeneration was altered in response to disuse remained unexplored in the former investigations.

Satellite cells are postnatal muscle stem cells that play a pivotal role in the regeneration of skeletal muscle following injury. Satellite cell populations are heterogeneous and may differ in features such as their myogenic differentiation predisposition, gene expression **profile**, lineage potential of nonmyogenic fates, and stemness. **Interestingly, different studies have also demonstrated modifications in satellite cell numbers in response to different stimuli and models of disuse muscle atrophy (Arentson-Lantz et al, 2016;Snijders et al, 2014;Suetta et al, 2013). On this basis, we focused the current investigation on the assessment of the regenerative**

potential and satellite cell numbers during recovery following immobilization of the hindlimb for seven days. This experimental approach enabled us to mimic a physiological condition (immobilization) compared to other models in which myotoxins have been used to trigger muscle regeneration following injury in mice. The seven-day time-point was chosen on the basis of previous studies (Chacon-Cabrera et al, 2016; Chacon-Cabrera et al, 2017a), in which early and late biological regeneration events could be identified. Furthermore, we also hypothesized that a differential pattern of myogenic precursor cell markers may be expressed in the satellite cells of the hindlimb muscles exposed to unloading and reloading, respectively. Accordingly, we sought to specifically investigate in the hindlimb muscles of mice exposed to seven-day periods of unloading and reloading: 1) muscle progenitor cell counts isolated from all the hindlimb muscles using fluorescent-activated cell sorting (FACS), 2) levels of satellite cells and regeneration events using histological (immunofluorescence, gastrocnemius and soleus) and electron microscopy analyses (gastrocnemius), and 3) to analyze the specific pattern of myogenic precursor cell markers in the satellite cell yields obtained from the FACS analyses. The model of disuse-induced muscle atrophy and recovery employed in the current investigation has been previously well-validated (Chacon-Cabrera et al, 2016; Chacon-Cabrera et al, 2017a).

MATERIALS AND METHODS

Animal experiments and Ethics approval

Female C57BL/6J mice (10 weeks old, weight ~20 g) were obtained from Harlan *Interfauna Ibérica SL* (Barcelona, Spain). Mice were kept under pathogen-free conditions in the animal house facility at Barcelona Biomedical Research Park (PRBB), with a 12:12 hour light: dark cycle.

Mice were exposed to unilateral hindlimb immobilization as previously

described to reproduce a model of disuse muscle atrophy (Chacon-Cabrera et al, 2016;Chacon-Cabrera et al, 2017a). Briefly, the left hindlimb was shaved with clippers and was enveloped using surgical tape. The hindlimb was introduced in a 1.5 mL microcentrifuge tube with cover and bottom lids removed, while maintaining the foot in a plantar-flexed position to induce the maximal atrophy of the target limb muscles: gastrocnemius, soleus, tibialis anterior (TA), extensor digitorum longus (EDL) and quadriceps femoris (QF). The entire procedure was carried out using mouse restrainers, without anesthesia. As the weight of the tube was approximately 0.6 g, it did not interfere with the usual mobility of the mice. In this study, for satellite cell isolation experiments a total number of 40 mice were employed. Animals were further subdivided into the two following groups: 1) 7-day immobilization group (7-day I, N=20/group) and 2) 7-day recovery group (7-day R, N=20/group), in which the splint was removed following the seven-day immobilization period to let the animals move freely in their cages in order to evaluate muscle recovery. The target samples were obtained by pooling together the gastrocnemius, soleus, TA, EDL and QF muscles from two different mice belonging to the same study group. The gastrocnemius, soleus, TA, EDL and QF from two different mice belonging to the contralateral hindlimb from the 7-day I or 7-day R groups of mice served as the control muscles. For the immunofluorescence and electron microscopy experiments a total number of 16 additional mice were employed (see below specific details for this type of experiments). The animals were also divided into two groups (7-day I and 7-day R mice, n=8/group). The gastrocnemius muscle was cut into two fragments that were used for immunofluorescence and electron microscopy analyses, respectively. Given the very small size of the soleus, only histological (immunofluorescence) analyses could be conducted on this muscle.

All animal experiments were conducted in the animal facilities at *Parc de Recerca Biomèdica de Barcelona* (PRBB). This controlled study was designed in accordance with the European Community Directive 2010/63/EU and Spanish Legislation (*Real Decreto* 53/2013, BOE 34/11370-11421, 2013) for the use and care of animals followed in our institution (PRBB animal facilities) and the Helsinki convention for the use and care of animals. Ethical approval was obtained by the Animal Research Committee (Animal welfare department in Catalonia, Spain, EBP-13-1485).

***In vivo* measurements in the mice**

In all the study animals, food intake was measured daily, and food and water were supplied ad libitum for the entire duration of the immobilization or recovery periods. In all mice, body weight and limb strength were determined on day 0, and right at the end of the immobilization or recovery time-points (day 7, as described above) using a scale and a grip strength meter (Bioseb, Vitrolles, France), respectively, as previously reported (Barreiro et al, 2016;Chacon-Cabrera et al, 2014;Chacon-Cabrera et al, 2015;Chacon-Cabrera et al, 2016;Chacon-Cabrera et al, 2017b;Chacon-Cabrera et al, 2017a). In the two groups of animals, total body weight and limb strength gain variables were calculated as the percentage of the measurements performed at the end of the study period (7-day I or 7-day R) with respect to the same measurements obtained at baseline (day 0 or on day 7 following the immobilization protocol) in the 7-day I and 7-day R study groups, respectively.

Sacrifice and sample collection

Mice from all experimental groups were sacrificed after the corresponding immobilization or recovery time-cohort. Each mouse was previously inoculated intraperitoneally with 0.1 mL sodium pentobarbital (60 mg/Kg). In all cases, the pedal

186 and blink reflexes were evaluated in order to verify total anesthetic depth. Animal
187 sacrifice took place following diaphragm extraction. For isolation of muscle cell
188 progenitor experiments the following muscles were obtained from all the animals at the
189 time of sacrifice: gastrocnemius, soleus, TA, EDL and QF (entire muscles in all cases).
190 As abovementioned, all study muscles from the hindlimb were pooled together by
191 groups of two for each experimental condition (7-day I and 7-day R) as well as from the
192 contralateral control hindlimb muscles, thus a total of 10 samples were analyzed in each
193 group (contralateral control muscles and both 7-day I and 7-day R mice). For
194 immunofluorescence and electron microscopy experiments, gastrocnemius and soleus
195 muscles were obtained at the time of sacrifice in each experimental cohort. As soleus
196 muscle was very small, only immunofluorescence analyses, but not electron
197 microscopy, were conducted (see below specific details). For stem cell progenitor
198 isolation experiments, the muscle samples were preserved in cold Dubelcco's Modified
199 Eagle's Medium (DMEM) to be immediately processed as described below. For
200 identification of several muscle regeneration markers, RNA was extracted from the
201 isolated satellite cell yields (see below).

202 **Satellite cell isolation using FACS**

203 A schematic representation of these methodologies is shown in Figure 1. Immediately
204 after dissection, muscles were processed following a modified version of a previously
205 described protocol (Pasut et al, 2012) (Figure 1). Pools of gastrocnemius, soleus, TA,
206 EDL and QF muscles were first minced with scissors and secondly with a razor blade
207 (Electron microscopy science, Hatfield, PA, USA). The minced muscles were collected
208 in a 50-mL tube containing 40 mL of cold DMEM to be subsequently washed to remove
209 fat tissue. Enzymatic digestion with a DMEM media containing 2.5 U/mL collagenase
210 (Serva Electrophoresis, Heidelberg, Germany) and 2.5 U/mL dispase (Sigma Aldrich,

211 Sant Louis, Missouri, USA) was immediately performed at 37°C in an agitation bath for
212 10 minutes.

213 The digestion procedure was repeated four times and the digested muscle
214 solution was then filtered through a 100-µm mesh filter (Corning, New York, NY,
215 USA). Immediately afterwards, the digestion was stopped by adding 2 volumes of 10%
216 fetal bovine serum (FBS) in phosphate buffered saline (PBS), and the muscle solution
217 was filtered through a 70-µm mesh filter (Corning). The filtered solution was
218 centrifuged at 300 x g for 5 minutes. The pellet was kept and the supernatant was
219 centrifuged again in order to recover the maximum amount of cells. Finally, the two cell
220 pellets were combined in a fresh tube to be re-suspended in FACS buffer [(1 mL PBS
221 solution containing 2.5% goat serum (Sigma Aldrich)]. The number of cells was
222 counted using a Neubauer chamber.

223 Prior to incubations with antibodies, the cells were washed in 20-mL cold
224 DMEM and centrifuged at 1,700 rpm for 10 minutes to recover the cell pellet. Cells
225 were resuspended in 1×10^6 cell/100 µL FACS buffer. All these cells were incubated
226 with antibodies used to specifically identify the satellite cells: Phycoerythrin (PE)-
227 conjugated anti-alpha-7 integrin (Ablab, Vancouver, Canada), a heterodimeric integral
228 membrane protein critical for the modulation of cell-matrix interactions, and
229 allophycocyanin (APC)-conjugated anti-CD34 (BD Pharmigen, San Jose, CA, USA), as
230 a cell surface sialomucin (a mucopolysaccharide molecule containing sialic acid) with
231 reported anti-adhesive, motile, and pre-proliferative properties for 30 minutes.
232 Additionally, the cells from both study groups of mice were incubated at the same time
233 (30 minutes) with specific antibodies to exclude other cell type populations that might
234 have also been present in the muscle extracts, namely endothelial cells, leukocytes and
235 hematopoietic cells: phycoerythrin (PE)-cyanine7 (Cy7)-conjugated anti-CD31

(Biolegend, San Diego, CA, USA), a marker of endothelial cells, both anti-CD11b (Biolegend) and anti-CD45 (Biolegend) as markers of leukocytes, and anti-Sca-1 (Biolegend), a marker of hematopoietic stem cells. The excluded cell populations were named negative-lineage [Lin (-)].

Subsequently afterwards, all the study cells were incubated with 4',6-diamino-2-phenylindol (DAPI) in order to exclude the dead cells (cells DAPI+ exclusively) five minutes prior to the start of FACS analyses (Figure 1, FACS Aria II SORP, BD Biosciences, San Jose, CA, USA). Once DAPI+ cells and other cell types (endothelial, leukocytes, and hematopoietic) were excluded, the cells identified using FACS were small and of round shape indicating a relatively low complexity. Cells stained for PE-conjugated anti-alpha-7 integrin and APC-conjugated anti-CD34 were sorted out and were named alpha-7 integrin+/CD34+ muscle progenitors (Figure 1). Muscle progenitor cells were expressed as the percentage of alpha-7 integrin+/CD34+ progenitors to the total numbers of isolated viable cells. These cells were specifically preserved following the sorting experiments to be further analyzed as described below.

Satellite cell RNA extraction

Total RNA was first isolated from the yield of sorted satellite cells using Trizol reagent following the manufacturer's protocol (Life technologies, Carlsbad, CA, USA). Total RNA concentrations were determined spectrophotometrically using the NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA).

Procedures of mRNA reverse transcription (RT)

A single RT was performed from which all the target genes of the study were analyzed. First-stranded cDNA was generated from mRNA using oligo(dT)₁₂₋₁₈ primers and the Super-Script III reverse transcriptase following the manufacturer's instructions (Life Technologies).

261 Quantitative real time-PCR amplification (qRT-PCR)

262 TaqMan based qPCR reactions were performed using the ABI PRISM 7900HT
263 Sequence Detector System (Life Technologies, Carlsbad, CA, USA) together with
264 commercially available gene expression assays. The probes corresponding to the
265 following genes involved in muscle regeneration were tested: *pax7* (Mn01354484_m1,
266 Life Technologies), myogenic differentiation 1 (*myoD*) (Mm00440387_m1, Life
267 Technologies), marker of cell proliferation ki-67 (*ki67*) (Mm01278617_m1, Life
268 Technologies) and myogenin (Mm00446194_m1, Life Technologies). The
269 housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)
270 (Mm99999915_g1, Life Technologies) served as the endogenous control for mRNA
271 gene expression. Reactions were run in duplicates, and mRNA data were collected and
272 subsequently analyzed using the sequence detection system relative quantification
273 software version 2.4 (Applied BioSystems), in which the comparative C_T method ($2^{-\Delta\Delta C_T}$)
274 for relative quantification was employed (Livak and Schmittgen, 2001).

275 Satellite cell identification using immunofluorescence microscopy

276 The protocol used in these experiments was similar to that published in previous studies
277 (Arentson-Lantz et al, 2016; Snijders et al, 2014; Suetta et al, 2013). Gastrocnemius and
278 soleus muscles were fixed in 4 % paraformaldehyde solution, pH 6.9 (EMD Millipore
279 corporation, Billerica, MA, USA) and were embedded progressively with increasing
280 concentrations of sucrose. They were subsequently embedded in tissue-tek optimal
281 cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) to be snap-
282 frozen in 2-methyl-butane immersed in liquid nitrogen as previously described (Barthel
283 and Raymond, 1990). Ten- μ m frozen sections were cut using a cryostat-microtome
284 (Leica CM3050S, Leica Biosystems, Wetzlar, Germany) at -20°C and were mounted on
285 glass slides. Immunofluorescence staining was used to detect satellite cells in both

286 quiescent and proliferative states using specific antibodies (see below). Briefly, muscle
287 cross-sections were air-dried for 30 minutes and were rinsed with PBS for another 15
288 minutes. PBS was used to rinse the sections among the different incubation steps. After
289 rinsing, the sections were put in cold methanol for six more minutes. Then, the sections
290 were boiled using a hot bath in 0.1 M citrate buffer (pH 6.0) for 12 minutes, and were
291 then blocked with 10% goat serum in PBS for two hours. Subsequently, sections were
292 incubated with Mouse IgG blocking reagent (MOM) (Vector Laboratories, Burlingame,
293 CA, USA) for 30 minutes. Afterwards, they were incubated overnight with a mixture of
294 two antibodies: mouse monoclonal anti-pax7 antibody (1:20; sc-81648, Santa Cruz
295 Biotechnology Inc., Dallas, TX, USA) and rabbit polyclonal anti-ki-67 antibody (1:100;
296 ab15580, Abcam, Cambridge, UK), prepared in an antibody solution (1% goat serum
297 dissolved in PBS, at 4°C). Anti-pax7 antibody was used to detect satellite cells, while
298 anti-ki-67 antibody was used to detect proliferating cells (namely blood cells and
299 proliferating myoblasts) (Arentson-Lantz et al, 2016;Snijders et al, 2014;Suetta et al,
300 2013). The double staining of cells with both anti-pax7 and anti-ki-67 simultaneously,
301 detected proliferating satellite cells. Following incubation with the primary antibodies
302 and after rinsing with PBS, the sections were incubated at room temperature with the
303 corresponding secondary antibodies for one hour: anti-mouse IgG1 Alexa Fluor 488
304 antibody and anti-rabbit IgG (H+L) Alexa Fluor Plus 555 (1:400 both, Invitrogen,
305 Thermo Fisher Scientific), also prepared in an antibody solution. Finally, the sections
306 were mounted using the fluorescent mounting medium DAPI G-Fluoromount medium
307 (Southern Biotech, Birmingham, AL, USA), which specifically stained DNA (allowing
308 identification of all nuclei) in the muscle sections. A fluorescence microscope (×40
309 objective, Nikon Eclipse Ni, Nikon, Tokyo, Japan) coupled with a digitizing camera
310 was used to identify and count the number of the satellite cells (10 fields) in each study

sample. In each muscle section from gastrocnemius and soleus of the two study groups of mice, results were expressed as the percentage of either pax7-positive, ki-67-positive, or both pax7- and ki-67 double-stained nuclei to the total number of counted myonuclei in the 10 fields.

Identification of satellite cells and regeneration events using electron microscopy

For these specific experiments only the gastrocnemius (larger size) could be used. Gastrocnemius muscles were gently excised into very small fragments. These pieces were then further cut into two 1 x 0.5 mm-fragments on a smooth surface using tweezers and a surgical blade. The muscle fragments were immediately immersed into a fixative solution containing 2% glutaraldehyde in cacodylate buffer (0.2M, pH 7.4) for 24 hours and were then post-fixed in 1% osmium tetroxide solution in the cacodylate buffer for one more hour. The samples were subsequently dehydrated in a graded series of ethanol from 70 to 100%, were then transitioned into the intermediate solvent propylene oxide, to be finally embedded with increasing concentrations of an Epon resin (until 100% of epon resin-embedding was reached). Subsequently, samples were embedded longitudinally in a mold containing fresh resin, and were polymerized at 60°C for 24 hours. Semithin sections of 0.4 µm each were then obtained using a glass knife, and were immediately stained with toluidine blue in order to identify the specific region to be thin-sectioned at 50-80 nm using a diamond knife (Diatome, Nidau, Switzerland) and an ultramicrotome (Ultracut E, Reichert-Jung, Austria). The thin sections were placed onto 250-mesh copper/nickel grids and were stained with uranyl acetate and lead citrate. Satellite cells were identified and counted using a transmission electron microscope (FEI CM100 TEM; Philips, Eindhoven, The Netherlands) with an attached Veleta digital camera (iTEM, Olympus, Tokyo, Japan) and iTEM software was used to capture the images (Davies, 1999). Satellite cells were identified as small round-

shaped cells located between the basal lamina and the sarcolemma of the myofibers. Fifty fields were counted in each study sample. The following parameters were assessed in the ultrastructural muscle preparations from the study muscle samples: 1) number of satellite cells, located between the basal lamina and the sarcolemma, in the measured area (mm^2), 2) early regeneration events characterized by the presence of internal myonuclei located in groups in the measured area (mm^2), and 3) late regeneration events identified as the presence of isolated internal myonuclei in the measured area (mm^2). Numbers of all these three ultrastructural events were counted in 50 fields of all the study samples and are illustrated in Table 2.

Statistical analysis

Results are presented as mean values (standard deviation). The normality of the study variables was explored using the Shaphiro-Wilk test. Food intake and percentage of change of both total body weight and limb strength variables are represented in Table 1, whereas the biological variables are represented in Table 2 and Figures (mean and standard deviation). The following statistical approaches were used in the study for different purposes. Firstly, the variables food intake and percentage of change of total body weight and limb strength variables in mice from the 7-day I cohort were compared with mice from the 7-day R cohort using the unpaired Student's T-test. Secondly, one-way analysis of variance (ANOVA) was used to assess potential differences in all the study biological variables among the three study groups of muscles samples: 7-day I, 7-day R and the control muscles from the contralateral hindlimb. Tukey post hoc analysis was used to adjust for multiple comparisons. A level of significance of $p < 0.05$ was established in the study. Comparisons of biological variables of the muscles obtained from the contralateral hindlimb showed no significant differences between the seven-day I and the seven-day R groups of mice. Thus, for the sake of clarity and conciseness,

361 this group has been named contralateral control muscles in the results below.

362

363 RESULTS

364 Physiological characteristics of the study animals

365 As shown in Table 1, food intake did not differ between the 7-day I and 7-day R mice.

366 The variables percentage of body weight and limb strength gain were significantly
367 increased in the 7-day R compared to 7-day I mice (Table 1).

368 Disuse reduces satellite cell numbers in muscles

369 The number of alpha-7 integrin+/CD34+ satellite cells of the 7-day I group was
370 significantly reduced compared to the contralateral control muscles (Figure 2). Alpha-7
371 integrin+/CD34+ satellite cell numbers were significantly higher in the 7-day R mice
372 compared to the 7-day I group, although they did not reach levels detected in the

373 contralateral control muscles (Figure 2). Immunofluorescence pax7-positive cells were
374 also significantly reduced in gastrocnemius and soleus muscles of the 7-day I animals
375 compared to the contralateral control muscles (Figures 3A-3D, respectively). Levels of
376 immunofluorescence pax7-positive cells were also lower in 7-day R muscles compared
377 to contralateral control muscles in both gastrocnemius and soleus muscles (Figures 3A-
378 3D, respectively). In the gastrocnemius, but not the soleus, numbers of proliferating
379 nuclei (ki-67 positively stained) were significantly higher in the 7-day R mice compared
380 to both 7-day I and contralateral control muscles (Figures 3A-3D). Additionally, in the
381 gastrocnemius, but not the soleus, specific proliferating satellite cells (both pax7 and ki-
382 67 double-stained) were significantly greater in the 7-day R group compared to
383 contralateral control muscles (Figures 3A-3D).

384 Ultrastructural analyses of satellite cells and muscle regeneration

385 Examples of the normal ultrastructure of muscle sections and of a satellite cell are

shown in Figures 4A-4B, respectively. Compared to contralateral control muscles, ultrastructural satellite cell counts were significantly greater in the gastrocnemius of the 7-day R mice, whereas no significant differences were seen in the muscles of the 7-day I animals (Table 2). Numbers of grouped internal nuclei (early regeneration phase) were significantly greater in the 7-day I and 7-day R mice compared to the contralateral control muscles (Table 2 and Figure 4C). Compared to contralateral control muscles, in the gastrocnemius, counts of isolated nuclei (marker of late regeneration phase) were significantly lower in the 7-day I mice, while they were significantly greater in the 7-day R animals (Table 2 and Figure 4D). Satellite cell fusion to existing myofibers was demonstrated in the gastrocnemius ultrastructural sections of the 7-day I mice (Figures 5A-C) and the 7-day R animals (Figure 5D). Sarcomeric membrane folding took place following satellite cell internalization (Figure 5B).

Disuse induces a specific pattern of regenerative markers in muscles

Levels of *ki67* gene expression were significantly higher in the 7-day R group compared to both 7-day I mice and the contralateral control muscles (Figure 6). *Pax7* and *MyoD* gene expression levels were significantly higher in the 7-day I mice compared to the contralateral control muscles, while the expression of these markers was significantly lower in hindlimb muscles from 7-day R animals compared to 7-day I mice (Figures 7A-7B, respectively). *Myogenin* expression did not significantly differ between the 7-day I and the contralateral control muscles, whereas a significant rise in this marker was observed in muscles of the 7-day R animals compared to both the 7-day I mice and the contralateral control muscles (Figure 7C).

DISCUSSION

In the study, a large-scale approach, in which satellite cells were isolated from all the

limb muscles using FACS, was followed. The rationale to use such methodologies was to identify a pure population of muscle progenitors as demonstrated in previous studies (Yin et al, 2013). **In fact**, one of the main goals in the study was to characterize the gene expression profile of the isolated progenitor cells during recovery following a period of hindlimb muscle disuse for seven days, **which was defined as a key time-point to analyze early and late events as previously shown in this experimental model (Chacon-Cabrera et al, 2016;Chacon-Cabrera et al, 2017a).** Furthermore, the use of methodologies such as histological procedures enabled us to define the localization and numbers of active and proliferating satellite cells in the study muscles. Besides, electron microscopy analyses also revealed that differential events of early and late regeneration phases took place in the muscles of the two study groups of mice.

In the current investigation, the study hypothesis has been confirmed to a great extent. **A specific program of muscle regeneration was induced during recovery following hindlimb immobilization.** The number of progenitor muscle cells was reduced in limb muscles isolated from mice exposed to a seven-day period of hindlimb disuse. **Histological analyses also revealed that pax7-positive satellite cells were reduced in both slow- and fast-twitch muscle types of the 7-day I mice.** Additionally, ultrastructural events of late muscle regeneration also decreased in the **gastrocnemius** of the latter animals. In fact, these findings are consistent with what has been found in other models of disuse, in which a significant decline in muscle satellite counts was demonstrated along with severe atrophy in young healthy subjects at rest (Arentson-Lantz et al, 2016;Mackey et al, 2009) and in experimental models of disuse muscle atrophy (Darr and Schultz, 1989;Mitchell and Pavlath, 2004;Wang et al, 2007). In elderly subjects, a decline in muscle satellite cells was also observed following immobilization for two weeks using a whole-leg casting (Suetta et al, 2013). Another

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study, however, revealed no significant changes in muscle satellite cell counts in the quadriceps muscle of healthy young individuals exposed to several weeks of one-legged knee immobilization using a cast (Snijders et al, 2014). Discrepancies among studies could be attributed to differences in the experimental models, age of the study subjects, previous activity of the target individuals, and/or methodologies used to identify the satellite cell numbers. A major strength and novelty in the current study was the use of three different approaches to identify satellite cells and muscle regeneration features in the mice exposed to unloading and reloading.

Importantly, removal of the splint (recovery period) induced a rise in the number of progenitor cells in the limb muscles of the mice during recovery. Although in these mice the content of satellite cells did not reach the baseline levels observed in the contralateral control muscles, it was significantly greater than in the seven-day I animals. These results imply that proliferation of muscle progenitors is a key step in the initiation of the muscle regeneration process during the recovery period. In line with this, numbers of proliferating satellite cells and ultrastructural cell counts were greater in the gastrocnemius of the seven-day R mice compared to control muscles as well as ultrastructural events of late muscle regeneration (isolated internal nuclei). In the soleus muscle, however, no differences were seen among the study groups probably due to the different fiber type composition of this muscle. These findings suggest that the fast-twitch gastrocnemius may have contributed to a great extent to the increased numbers of progenitor cells detected in the seven-day R muscles during the FACS analyses.

Importantly, in the animals, reestablishment of normal muscle activity following a resting period may mimic what happens in response to exercise training in humans. As such, strength training was also shown to induce a significant increase in satellite cell numbers along with the expression of myogenic regulatory and growth factors in the

quadiceps muscle of healthy young subjects (Hanssen et al, 2013).

Levels of proliferating cells in general as measured by the ki-67 marker were only significantly increased in muscle progenitor cells in the animals exposed to seven-day recovery compared to levels detected in the control muscles and the seven-day immobilized mice. As abovementioned, numbers of proliferating satellite cells identified as those positively stained for both pax7 and ki-67 antigens were also greater in the gastrocnemius of seven-day R mice. These findings are in line with those previously reported in which counts of active satellite cell as identified exclusively by ki-67 expression levels also increased following muscle activity in healthy humans (Mackey et al, 2009). Interestingly, the rise in ki-67 expression also corresponded to that seen in the muscle progenitor cells during recovery. It should be mentioned that in the current study, proliferating satellite cells were detected using two antibodies since other cell types (e.g. hematopoietic cells) might have also proliferated during the recovery phase in the study muscles. We considered this to be a more accurate approach than that exclusively based on ki-67 staining.

Characterization of the muscle progenitors from a molecular standpoint has revealed that the expression of markers such as a *pax7* and *myoD* was upregulated in the muscle progenitors of mice during unloading, while it reached baseline levels during reloading. These findings suggest that despite the decrease in muscle progenitors observed during unloading, satellite cells were not in G₀ phase as *myoD* is expressed in myogenic precursor cells but not in quiescent satellite cells (Yin et al, 2013). The current observations are in line with those reported in models of disuse muscle atrophy, in which the expression of markers of satellite cell activation was upregulated, while satellite cell counts were also reduced (Arentson-Lantz et al, 2016; Snijders et al, 2014; Suetta et al, 2013).

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3 486 The process of satellite cell activation during skeletal muscle injury including
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5 487 unloading is rather complex. For instance, periods of satellite cell proliferation and
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7 488 fusion to the existing fiber syncytium were demonstrated in the gastrocnemius of mice
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9 489 exposed to hindlimb suspension (Ferreira et al, 2006). In the present investigation,
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11 490 fusion of satellite cells was also observed in the gastrocnemius of muscles exposed to
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13 491 immobilization for seven days (Figure 5). On this basis, it would be possible to
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15 492 conclude that activation of satellite cells should have taken place early during
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17 493 unloading, thus, leading to the fusion of those cells to the differentiated myofibers
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19 494 immediately afterwards. These findings most likely account for the decrease in muscle
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21 495 progenitors seen in the 7-day I mice as revealed by both the FACS (all hindlimb
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23 496 muscles) and histological analyses (gastrocnemius). Furthermore, ultrastructural events
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25 497 of early regeneration (grouped internal nuclei as a marker of myofiber fusion) were,
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27 498 indeed, identified and increased in the mice exposed to seven-day I compared to control
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29 499 muscles. Collectively, these observations are also consistent with the decrease in *pax7*
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31 500 and *myoD* mRNA expression levels detected in the hindlimb muscles of animals during
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33 501 recovery in the present study. Moreover, mRNA expression levels of *myogenin* were
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35 502 upregulated in the latter muscles, which implied that during reloading, terminal
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37 503 differentiation was underway following the required decline in the expression of
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39 504 satellite cell activation markers (Yablonka-Reuveni and Rivera, 1994).
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45 505 **Conclusions**

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47 506 Recovery following hindlimb immobilization was characterized by a specific program
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49 507 of muscle regeneration (cellular and molecular) events. Hindlimb immobilization
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51 508 induced a decline in muscle progenitor cells together with an upregulation of markers of
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53 509 satellite cell activation, suggesting that fusion to existing myofibers takes place during
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55 510 unloading as demonstrated by the ultrastructural analyses. Muscle recovery as a result
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3 511 of splint removal induced a significant rise in muscle precursor cells and regeneration
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5 512 events along with a reduction in the expression of satellite cell activation markers and a
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7 513 concomitant rise in terminal muscle differentiation expression. These are novel, relevant
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9 514 findings of potential applicability for the treatment of disuse muscle atrophy, which is
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For Peer Review

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FIGURE LEGENDS

Figure 1: Representative flow-chart of the satellite cell isolation procedures using FACS analyses. The different steps are represented in the different boxes. The flow of the protocol is represented by dark thick arrows. Exclusion of the unwanted cells is represented by the corresponding grey thin arrows. Definition of abbreviations: FACS, fluorescence-activated cell sorting; DAPI, 4',6-diamino-2-fenilindol; Lin(-), negative lineage.

Figure 2: Mean values and standard deviation of the percentage of alpha-7 integrin+/CD34+ satellite cells in the contralateral control muscles (N=10/group), and both the 7-day I (N=10/group) and 7-day R (N=10/group) cohorts of mice. Definition of abbreviations: I, immobilization; R, recovery. Statistical significance is represented as follows: *, $p < 0.05$ and ***, $p < 0.001$ between hindlimb muscles from either 7-day I or 7-day R mice and the contralateral control muscles; #, $p < 0.05$ between muscles from the 7-day I and 7-day R groups of animals.

Figure 3: Mean values and standard deviation of satellite cell counts as identified by the number of pax7-positive cells (N=8/group), ki-67-positive cells (N=8/group), and pax7+ki-67-positive cells (N=8/group) in (A) gastrocnemius and (B) soleus muscles of the contralateral control muscles (open bars), the 7-day I (black bars), and 7-day R (grey bars) cohorts of mice. Representative images of immunofluorescence staining of DAPI (left panels), pax7 (middle-left panels), ki-67 (middle-right panels) and cells positively stained for both pax7 and ki-67 markers (right panels) in (C) gastrocnemius and (D) soleus muscles of the contralateral control muscles, the 7-day I and the 7-day R cohorts of mice. Thin arrows indicate pax7 positive cells, notched arrows indicate ki-67 positive cells and triangle arrows indicate double-stained nuclei for both pax7 and ki-67 positive cells (proliferating satellite cells). Definition of abbreviations: I, immobilization; R,

recovery. Statistical significance is represented as follows: *, $p < 0.05$ and **, $p < 0.01$ between hindlimb muscles from either the 7-day I or the 7-day R animals and the contralateral control muscles, ^{##}, $p < 0.01$ between muscles of the the 7-day I and 7-day R mouse groups.

Figure 4: Representative image of (A) a normal myofiber with two peripheral myonuclei (thick black arrows) in gastrocnemius muscle obtained from a 7-day R mouse, (B) a normal satellite cell in gastrocnemius from contralateral control muscles (thick black arrows) located between the basal lamina (dashed arrow) and the sarcolemma, (C) muscle in early regeneration phase in the gastrocnemius of a 7-day I mouse characterized by the presence of grouped internal myonuclei (thick black arrow), and (D) muscle in late regeneration phase in gastrocnemius muscle of a 7-day R mouse characterized by the presence of an isolated internal myonucleus (thick black arrow).

Figure 5: Representative images of satellite cell fusion showing grouped internal myonuclei (thick black arrows), indicative of recent fusion of satellite cells to existing myofibers following proliferation in the gastrocnemius obtained from (A-C) 7-day I mice and (D) 7-day R mice. Image shown in B corresponds to the enlargement of the circled region marked in A micrograph, in which sarcomeric membrane folding took place following satellite cell internalization.

Figure 6: Mean values and standard deviation of gene expression of *ki67* (cell proliferation marker) in the contralateral control muscles (N=10/group) and in muscles from the 7-day I (N=10/group) and 7-day R (N=10/group) mouse cohorts. Definition of abbreviations: I, immobilization; R, recovery. Statistical significance is represented as follows: **, $p < 0.01$ between hindlimb muscles from the 7-day R and the contralateral control muscles; ^{##}, $p < 0.01$ between muscles from the 7-day I and the 7-day R groups of mice.

Figure 7: Mean values and standard deviation of gene expression of the markers (A) *pax7*, (B) *myoD* and (C) *myogenin* in the **contralateral** control muscles (**N=10/group**) and in hindlimb muscles from the 7-day I (**N=10/group**) and 7-day R (**N=10/group**) **mouse cohorts**. Definition of abbreviations: I, immobilization; R, recovery. Statistical significance is represented as follows: *, $p < 0.05$ between **hindlimb muscles from either** the 7-day I or the 7-day R groups of mice and the **contralateral** control muscles; #, $p < 0.05$ and ##, $p < 0.01$ **between muscles from** the 7-day I and the 7-day R groups of mice.

Table 1. Characteristics of the study animals

	7-day I mice, n=20	7-day R mice, n=20
Food intake (grams/24h)	+4.7 (0.6)	+4.8 (0.4) n.s.
Total body weight, % gain	-3.7 (4.5)	+3.0 (4.7)***
Limb strength gain, % gain	-12.9 (7.6)	+16.2 (13.5)**

Data are presented as mean values (standard deviation).
Total body weight and limb strength gain variables were calculated in each animal group as described in Methods. Comparisons were made between the two groups of mice for each study variable (see statistical analyses in Methods section).
Definition of abbreviations: I, immobilization; R, recovery. Statistical significance is represented as follows: n.s., non-significant, **, p<0.01, and ***, p<0.001 between 7-day I and 7-day R mice.

Table 2. Ultrastructural satellite cell identification and counts in the gastrocnemius of all study groups of mice

	Contralateral control muscles (n=8)	7-day I mice (n=8)	7-day R mice (n=8)
SC counts (SC/mm²)	13.1 (6.3)	18.2 (6.9)	22.4 (6.4) **
Muscle in early regeneration phase (grouped internal myonuclei/mm²)	3.6 (2.9)	7.7 (3.3)*	7.6 (5.4)*
Muscle in late regeneration phase (isolated internal myonuclei/mm²)	10.7 (3.2)	5.9 (2.7)*	16.7 (4.4) **###

Data are presented as mean values (standard deviation).

Definition of abbreviations: I, immobilization; R, recovery; SC, satellite cells. Statistical significance is represented as follows: n.s., non-significant, *, $p < 0.05$ and ** $p < 0.01$ between either 7-day I or 7-day R mice, respectively, and the contralateral control muscles; ###, $p < 0.001$ between 7-day I and 7-day R animals. See Methods for detailed information on the quantification procedures of the study parameters.

Guitart et al. Figure 1

Figure 1

254x190mm (300 x 300 DPI)

Guitart et al. Figure 2

Figure 2

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Guitart et al. Figure 3

Figure 3 A-B

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Figure 3C

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Figure 3D

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Guitart et al. Figure 4

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Guitart et al. Figure 5

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Guitart et al. Figure 6

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Guitart et al. Figure 7

Figure 7

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