Muscle Precursor Cells enhance the functional Muscle Recovery and show synergistic effects with post-injury treadmill exercise in a muscle injury model in rats.

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ABSTRACT:

Background: Skeletal muscle injuries represent a major concern in sports medicine. Cell therapy has emerged as a promising therapeutic strategy for muscle injuries, although the preclinical data are still inconclusive and its potential clinical use has not yet been established.

Purpose: To evaluate the effects of Muscle Precursor Cells (MPCs) on muscle healing. MPCs were administered intramuscularly at 36 h after injury either alone or in combination with 2-weeks daily post-traumatic exercise training in a rat muscle injury model that mimics skeletal muscle lesions seen in athletes.

Study Design: Controlled laboratory study.

Methods: A total of 27 rats were used in the study. MPCs were isolated from rat (N=3) medial gastrocnemius muscles and expanded in primary culture. Skeletal muscle injury was induced in 24 rats and the animals were assigned to 3 groups. Injured animals received treatment based on a single ultrasound-guided MPCs (10^5 cells) injection (Cells group), or the MPCs injection in combination with 2-weeks daily exercise training (Cells-Exercise group). Animals receiving intramuscular vehicle injection were used as controls (Vehicle group). Muscle force was determined 2 weeks after muscle injury and muscles were collected for histology and immunofluorescence evaluation.

Results: Red fluorescence-labeled MPCs were successfully transplanted in the site of the injury by ultrasound-guided injection and localized in the injured area after 2 weeks. Transplanted MPCs participated in the formation of regenerating muscle fibers as corroborated by the co-localization of red fluorescence with dMHC-positive myofibers by immunofluorescence analysis. A strong beneficial effect on muscle force recovery was detected in Cells and Cells-Exercise groups (102.6% ± 4.0% and 101.5% ± 8.5% of maximum tetanus force (TetF) of the injured vs healthy contralateral muscle, respectively) compared to control (vehicle) animals (78.2% ± 5.1%). Both Cells and Cells-Exercise treatments stimulated the growth of newly formed regenerating muscles fibers, as determined by the increase in myofiber cross-sectional area (612.3 ± 21.4 and 686.0 ± 11.6 μm^2, respectively) compared to vehicle-injected animals

(247.5 ± 10.7 μm^2), that was accompanied by a significant reduction of intramuscular fibrosis in Cells and Cells-Exercise treated animals (24.2% ± 1,.3% and 26.0% ± 1.9% of collagen type I deposition, respectively) with respect to control animals (40.9% ± 4.1% in Vehicle group). MPCs-treated muscles induced a robust acceleration of the muscle healing process as demonstrated by the decreased number of dMHC-positive regenerating (enhanced replacement of developmental myosin isoform by mature myosin isoforms) myofibers (4.3% ± 2.6% and 4.1% ± 1.5% in the groups Cells and Cells-Exercise, respectively) compared to the Vehicle group (14.8% ± 13.9%).

Conclusion: Single intramuscular MPCs administration improves histological outcome and force recovery of the injured skeletal muscle in a rat injury-model which imitates the sports-related muscle injuries. Cell therapy showed a synergistic effect when combined with early active rehabilitation-protocol in rats, and suggests that a combination of treatments can generate novel therapeutic strategies for the treatment of human skeletal muscle injuries.

Clinical Relevance: Our study demonstrates the strong beneficial effect of MPC transplantation and the synergistic effect when the cell therapy is combined with early active rehabilitation-protocol for muscle recovery in rats, and opens new avenues for the development of effective therapeutic strategies for muscle healing and clinical trials in athletes undergoing MPC transplantation and rehabilitation protocols.

Keywords: Skeletal muscle injury; rat model; MPCs; cell therapy; physical exercise therapy; muscle healing.

What is known about the subject: Cell therapy has been postulated as a promising therapeutic strategy for tissue regeneration and healing. However, few pre-clinical studies have investigated the effects of intramuscular MPCs transplantation and exercise as potential therapeutic approaches for muscle healing and there is a lack of consistent pre-clinical data on the MPCs-therapy alone or in combination with the rehabilitation protocols to support the translation of MPCs-therapy to the clinical use in humans.

What this study adds to existing knowledge: We have addressed the individual effects of intramuscular MPC-injection and its combination with early active rehabilitation in a well-characterized experimental skeletal muscle injury-model that closely mimics injuries seen in human athletes. Our study demonstrates the efficacy of the intramuscular MPCs administration in the healing of injured muscle and reveals that combining it with early active rehabilitation provides synergistic effects on muscle recovery. Our study can hopefully help to translate the cell therapy in combination with rehabilitation protocols to the human translational studies.

INTRODUCTION

Skeletal muscle injuries are the most common sports-related injuries, accounting for up to 55% of all sports injuries and quite possibly impacting all musculoskeletal traumas [6,17,52]. Although skeletal muscle demonstrates a remarkable regeneration capacity after injury [38,40], the healing process is slow and often results in incomplete functional recovery and an increased risk for recurrence [25]. Upon injury, the adult muscle tissue activates a complex muscle regeneration process which is supported by distinct skeletal muscle stem cell populations, namely classical satellite cells (SCs) and another, more recently identified stem cell population, Muscle-Derived Stem Cells (MDSCs) [4,10,51]. MDSCs are defined as cells that possess the ability to produce both new muscle stem cells as well as myoblast and myofiber progeny, without themselves expressing markers of muscle differentiation. They possess the ability to differentiate into other cell lineages and have high myogenic capacity to effectively regenerate both skeletal and cardiac muscle. MDSCs can be stimulated to proliferate and fuse with surrounding myoblasts or pre-existing myofibers to regenerate damaged muscle tissue and recover muscle functionality [4,37,58].

The transplantation of stem cells into injured tissue in hopes to enhancing the repair process has long been a central goal of regenerative medicine and tissue engineering. This novel therapeutic strategy consists of the external supply of progenitor cells to the injured tissue to increase the number of cells that promote the tissue regeneration. This approach has been previously tested in the experimental transplantation of myoblasts in animal models of muscle injury and muscular dystrophy [5,8,16,40,48,55]. Both SCs and MDSCs have been identified as potential candidates for cell therapy of muscle diseases [15,35], and their translation for clinical use in humans is very plausible scenario, since they have the advantage that they can be isolated using a safe and minimally invasive skeletal muscle biopsy procedure. In addition, the autologous use of MDSCs has already been evaluated in different clinical trials for the treatment of urinary and fecal incontinence are ongoing [9,18,51], although their use in clinical trials for the treatment of

skeletal muscle injuries has not yet been initiated. Therefore, to date, only few preclinical studies have investigated the use of MDSCs for the treatment of acute skeletal muscle injury [4,27,34,39,42,53]. Based on previously described methodologies[3,19,36,47,59], we obtained a cell population of muscle precursor cells (MPCs) with slow adhesion cell characteristics. This stem cell population, including both SCs and MDSCs, can significantly improve muscle cell-mediated therapies[43] for the regeneration of injured skeletal muscle by taking advantage of the healing properties of various types of muscle stem cells present in skeletal muscle tissue.

Our purpose was to evaluate the potential clinical application of MPCs therapy alone and in combination with ideal rehabilitation protocol to determine whether the MPC transplantation improves the healing of the injured skeletal muscle. Thus, we investigated the effects of ultrasound-guided MPCs transplantation into the site of the injury, and the potential synergistic effect of combining MPCs transplantation with early, active rehabilitation protocol after injury, by using a well-characterized experimental skeletal muscle injury-model in rats which reproduces the injuries seen in human athletes [13,14]. To do this, we designed a preclinical study to evaluate the individual effects of MPCs transplantation alone or in combination with early active rehabilitation in our rat muscle injury-model.

METHODS

Animals, Surgery, and *in vivo* MPCs transplantation

Twenty-four male (8-week-old) Wistar rats (Harlan) were maintained at 22ºC with a 12:12 h light-dark cycle with water and food *ad libitum*. All procedures were performed in accordance with national (Royal decree 53/2013) and European (2010/63/UE) legislation. All animals followed a running treadmill training protocol during a 2-week training period, as previously described [14]. After 2 weeks of pre-injury training, animals were subjected to surgically-induced skeletal muscle injury in the medial gastrocnemius muscle as described [13]. Briefly, rats were anaesthetized with a mixture of ketamine (75 mg/kg, intraperitoneally [ip]) and xylazine (10 mg/kg, ip) before the surgical procedure. The muscle injury was generated by using an 18-gauge biopsy needle (Bard Monopty Disposable Core Biopsy Instrument, Bard Biopsy Systems). A transverse biopsy procedure was performed at the myotendinous junction level of the right leg medial gastrocnemius muscle (3 mm from the start of muscle-tendon junction and 2 mm in depth), resulting in a muscle injury of approximately 20-30% of the total cross-sectional area of the medial gastrocnemius muscle. Postsurgical analgesia (buprenorphine 0.01 mg/kg) was subcutaneously administered to all operated animals.

Rats were randomly assigned into three groups after skeletal muscle injury (N = 8 per group):

1. Vehicle group (Vehicle): A single ultrasound (US)-guided intramuscular injection of 30 µl MEM (Minimum Essential Medium; Gibco) in the site of the lesion was administered at 36 hours after injury.

2. Cell therapy group (Cells): A single US-guided intramuscular injection of cells suspension (1 x 10^5 MPCs resuspended in 30 µl MEM) was administered at 36 hours after the injury at the site of the muscle lesion.

3. Cell therapy and exercise group (Cells-Exercise): Rats were subjected to a combined treatment of a single US-guided intramuscular injection of cells suspension (1 x 10^5 MPCs resuspended in 30 µl MEM) administered at 36 hours after injury and in combination with a

daily treadmill exercise protocol for 2 weeks [14]. Cell dose was chosen based on previous studies of muscle stem cells transplantation into muscle injury models in rodents [4,34,39].

All US procedures were performed by using a portable MyLab ONE US device (Esaote SpA) and a SL3116 22MHz Linear transducer (Esaote SpA). An experienced radiologist in musculoskeletal ultrasonography carried out the intramuscular US-guided injections in the rat model (Supplementary video 1).

To prevent immune rejection of implanted MPCs, all rats were subjected to daily oral gavage administration of Tacrolimus (1.0 mg/kg/day, Cinfa) in combination with Mycophenolate mofetil (20 mg/kg/day, Mylan) for 15 days, starting the immunosuppressive treatment one day before muscle injury. The combination of Tacrolimus with Mycophenolate mofetil has already been demonstrated as an optimal method to suppress potential graft rejection in *in vivo* myoblast transplantation in muscle injury models in rodents[7,49,50]

MPCs isolation and culture

MPCs were isolated from rat medial gastrocnemius muscles (N=3) according to previously method described by Allen et al [3] and incorporating the modifications made by Zwetsloot et al.[59] and Machida et al. [36] Briefly, animals were euthanized by CO_2 inhalation and excised muscles were pooled, trimmed of excess connective tissue and fat and minced in sterile PBS (Phosphate-buffered saline; HyClone) containing 5 μg/ml Amphotericine B solution (Sigma-Aldrich). MPCs were isolated by enzymatic digestion of muscle fragments by incubating with 1.25 mg/ml of protease type XIV (Sigma-Aldrich) dissolved in DMEM medium containing 25 mM glucose and 1% antibiotic-antimycotic for 1 h at 37ºC. The digested muscle tissue was then centrifuged at 1,500 xg for 5 min, resuspended in 10 ml of warm PBS and centrifuged at 500 xg for 10 min. The collected supernatant were filtered through a 100 μm cell strainer (Corning), centrifuged at 1,500 xg for 5 min, and the MPCs pellet was resuspended in the growth medium Ham's F-10 nutrient mixture (Sigma-Aldrich) containing 20% FBS (Fetal bovine serum; Gibco), 1% CEE (Chicken Embryo Extract; Seralab) and 1% antibiotic-antimycotic (Gibco). MPCs were cultured using a pre-plating technique [36,47] and the cells were first seeded for 24 h on tissue culture-

treated 60 mm plates (Corning). After 24 h, the supernatant was collected and seeded onto a Matrigel-coated (1 mg/ml; Matrigel Basement Membrane Matrix, BD Bioscience) 60 mm culture plate. All cell culture experiments were performed in a humidified incubator with 5% CO_2 at 37ºC (NuAire).

Cell growth analysis

Primary cultured rat MPCs were trypsinized and counted in triplicates by performing the trypan blue (Sigma-Aldrich) exclusion test of cell viability using a Neubauer counting chamber (Thermo Fisher Scientific) under optical microscopy (Olympus IX71 microscope).

Muscle cells differentiation assay

For the differentiation experiments, MPCs were seeded on a Matrigel-coated 60 mm plate to 70-80% confluency in growth medium. Then, cultured cells were incubated with differentiation medium (DMEM:MEM in proportion 3:1, containing 2% HS (Horse serum; Gibco) and 1% antibiotic-antimycotic) for 10 days to induce cell fusion. Culture medium was daily changed during the differentiation procedure. All microphotographs and videos were taken using an Olympus IX71 microscope equipped with a DP70 camera (Olympus).

Cell transfection to express the fluorescent DsRed2 marker gene

To enable tracking of the cells after implantation into the muscle, MPCs were infected with the retroviral vector pIRES2-DsRed2 (Clontech) containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the Multiple Cloning Site and the Discosoma sp. red fluorescent protein (DsRed2) coding region. MPCs were incubated with 10 m.o.i (multiplicity of infection) lentiviral particles dissolved in DMEM with 25 mM glucose for 24 h at 37°C. After infection, DsRed2-positive cells were identified by fluorescence microscopy using a BX-61 microscope (Olympus) equipped with a DP72 camera (Olympus) and CellSens Digital Imaging software (version 1.9).

Measurement of Skeletal Muscle Force

Details of evaluation of skeletal muscle contractile function were previously described [13]. In brief, rats were anesthetized by an intraperitoneal injection of a mixture of ketamine:xylazine (75:10 mg/kg). Animals were placed in prone position in a platform to immobilize the limb and Achilles tendon was separated from the calcaneus and, after extracting the soleus muscle from the triceps surae to analyze the muscle strength of the gastrocnemius muscle, the tendon was attached to a force transducer (MLT 1030/D, ADInstruments) connected to a PowerLab/16SP data acquisition hardware (ADInstruments). The sciatic nerve was then exposed through a lateral incision on the thigh and connected to an electrode and a stimulator (Stimulus Isolator, FE180; ADInstruments). Muscle force analysis was determined at a constant room temperature of 25º-27ºC in both right (injured) and left (control) gastrocnemius muscles of each animal. Maximum tetanus force (TetF) was induced by a train of stimuli (100 Hz, 0.1 ms pulse width and 5 V) and analyzed using the LabChart v7 software (ADInstruments). Finally, the animals were euthanized by anesthetic overdose and gastrocnemius muscles were excised, weighed, frozen in supercooled 2-methylbutane (Alfa Aesar) and stored at -80ºC until further analysis.

Histological and Immunofluorescence Analysis

Frozen gastrocnemius muscles were transversely sectioned (10 µm thick) using a Cryotome (Leica) at below -20ºC and mounted on Poly-L-lysine-coated glass slides (VWR). Consecutive frozen sections were used for histological and immunofluorescence analysis. For histology, muscle sections were stained with Harris's hematoxylin (Casa Álvarez) and eosin (Panreac) and mounted with DPX mounting medium and a coverslip (VWR). For immunofluorescence analysis, frozen muscle sections were fixed in cold (-20ºC) acetone (Sigma-Aldrich) and immunostained with the primary antibodies rabbit anti-collagen-I (Abcam) and mouse anti-dMHC (developmental myosin heavy chain; Novocastra), and with secondary antibodies Alexa Fluor 488 anti-mouse or Alexa Fluor 488 anti-rabbit (Invitrogen). Slides were mounted with a coverslip and Fluoromount-G mounting medium with DAPI (Southern Biotech).

Analysis of Muscle Fiber Cross-sectional Area, dMHC, and Collagen-I Expression

For cross-sectional area (CSA) determination, a total of 200 to 300 fibers per muscle in each group were counted and measured in collagen type I immunofluorescence microphotographs. All analyzes and measurements were performed in blinded fashion by the same observer in order to avoid bias in the results. Area of muscle fibers was calculated based on a ratio of calibrated pixels to actual size (μm). dMHC expression levels were analyzed by measuring the average signal intensity in dMHC immunofluorescence microphotographs, presented as the percentage of dMHC-positive area respective to the total area of the image. The degree of fibrosis was evaluated in collagen type I immunofluorescence images by determining the area of collagen type I respective to the total area in microphotographs. Three images were randomly selected within the injured area of every gastrocnemius muscle sample by using a BX-61 microscope (Olympus) equipped with a DP72 camera (Olympus) and CellSens Digital Imaging software (version 1.9). CSA determination, the signal intensity in dMHC and the percentage of collagen-I area, were measured using the Image J software (version 1.46; National Institutes of Health).

Western blot analysis

For immunoblotting analysis, twenty consecutive frozen transverse sections (10 μm thick) of every gastrocnemius muscle at the level of the muscle injury were collected using a Cryotome (Leica) at below -20ºC. Skeletal muscle tissue samples were resuspended in RIPA buffer (20 mM sodium phosphate, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 0.1% β-glycerophosphate and complete protease and phosphatase inhibitor cocktails (Roche Diagnostics) and homogenized using an Omni TH tissue homogenizer (Omni International). Samples were centrifuged at 10,000xg at 4ºC for 15 min and the supernatants were used for Western blot analysis. Protein was quantified by the BCA protein assay (Pierce) and equal amounts of protein from supernatants were electrophoresed on 8% SDS gels and sequentially transferred to nitrocellulose (Biorad) membranes. After blocking with PBS (pH 7.4) containing 5% dry milk for 1 h at room temperature, membranes were incubated with

either 1:500 rabbit anti-dMHC (8087, ProSci Inc.), 1:500 rabbit anti-collagen type I (ab34710, Abcam) and 1:2000 mouse anti-α-Tubulin (T5168, Sigma) antibodies in PBS containing 5% dry milk at 4ºC for 16 h and washed three times for 10 min with PBS and 0.05% Tween-20 (Dako). Then, membranes were incubated with either HRP-conjugated secondary anti-rabbit IgG (NA934, Amersham) or anti-mouse IgG (NA931, Amersham) antibody in PBS containing 5% dry milk for 1 h at room temperature and washed three times for 10 min with PBS and 0.05% Tween-20. Chemiluminescence was detected using the Clarity™ chemiluminescence kit (Biorad) and bands were visualized in an Odyssey Fc (LI-COR Biosciences) detector and quantified using the Image J software (version 1.46; National Institutes of Health).

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics version 20.0 (IBM). The Shapiro-Wilk test was used to evaluate normality and homoscedasticity of the data. Paired Student's t-tests were used to determine statistical significance in comparisons of TetF between right (injured) and left (control) gastrocnemius muscle in every animal. Differences between right and left gastrocnemius muscle mass of every animal were also evaluated by paired Student's t-test. One-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (post hoc Holm-Sidak test) was performed to assess the differences in body mass, muscle mass, collagen-I percentage and TetF (expressed as percentage of injured vs control muscle) between the 3 groups of animals. A Kruskal-Wallis test was used to evaluate statistical significance in nonparametric comparisons between the different groups of animals for myofiber CSA and dMHC expression. In case of significant differences, a Mann Whitney U test was used to compare the different groups. Data from dMHC and collagen-I expression in western-blot analysis were normalised to tubulin and compared between groups by Kruskal-Wallis and Mann Whitney U tests. Differences were considered significant with a P value less than 0.05.

RESULTS

Muscle and Body Mass

No significant difference in body mass was observed between the study groups at the beginning or at the end of the experiments (Table 1). In addition, no significant difference was detected in gastrocnemius muscle mass either intra- or inter-groups of treatment.

	Body Mass (g)		Muscle Mass (g)	
	Initial Body Mass	Final Body Mass		Final GM Mass
Vehicle Group	268.3 ± 20.7	327.7 ± 22.9	Control leg Injured leg	2.1 ± 0.2 2.0 ± 0.2
Cells Group	294.3 ± 19.7	347.47 ± 19.0	Control leg Injured leg	2.3 ± 0.2 2.3 ± 0.1
Cells+Exercise Group	283.1 ± 19.7	320.3 ± 22.5	Control leg Injured leg	2.2 ± 0.1 2.2 ± 0.2

Table 1. Body weight and gastrocnemius muscle mass. Anesthetized animals were weighed just before to induce gastrocnemius muscle injury and at the end of the treatments. Right (injured) and left (control) gastrocnemius muscle (GM) were excised and weighed from all the animals just before euthanasia at the end of the treatment period. Values are presented as mean ± SD.

Proliferation and differentiation of primary cultured rat MPCs

MPCs were isolated from rat medial gastrocnemius muscles and cultured *in vitro*. Rat MPCs exhibited spindle-like shape after 3 days in primary culture (Figure 1A, left panel). Four days later the cell density was significantly increased, more than 4-fold (Figure 1B). In order to confirm the myogenic properties of the primary cultured MPCs before *in vivo* transplantation, cells were grown to confluence and induced to differentiate by replacing the proliferation medium with differentiation medium (Figure 1A, right panel). After 5-7 days in differentiation medium, MPCs demonstrated the capacity to fuse and form polinucleated myotubes with spontaneous contractile properties (Figure 1A; Supplementary video 2).

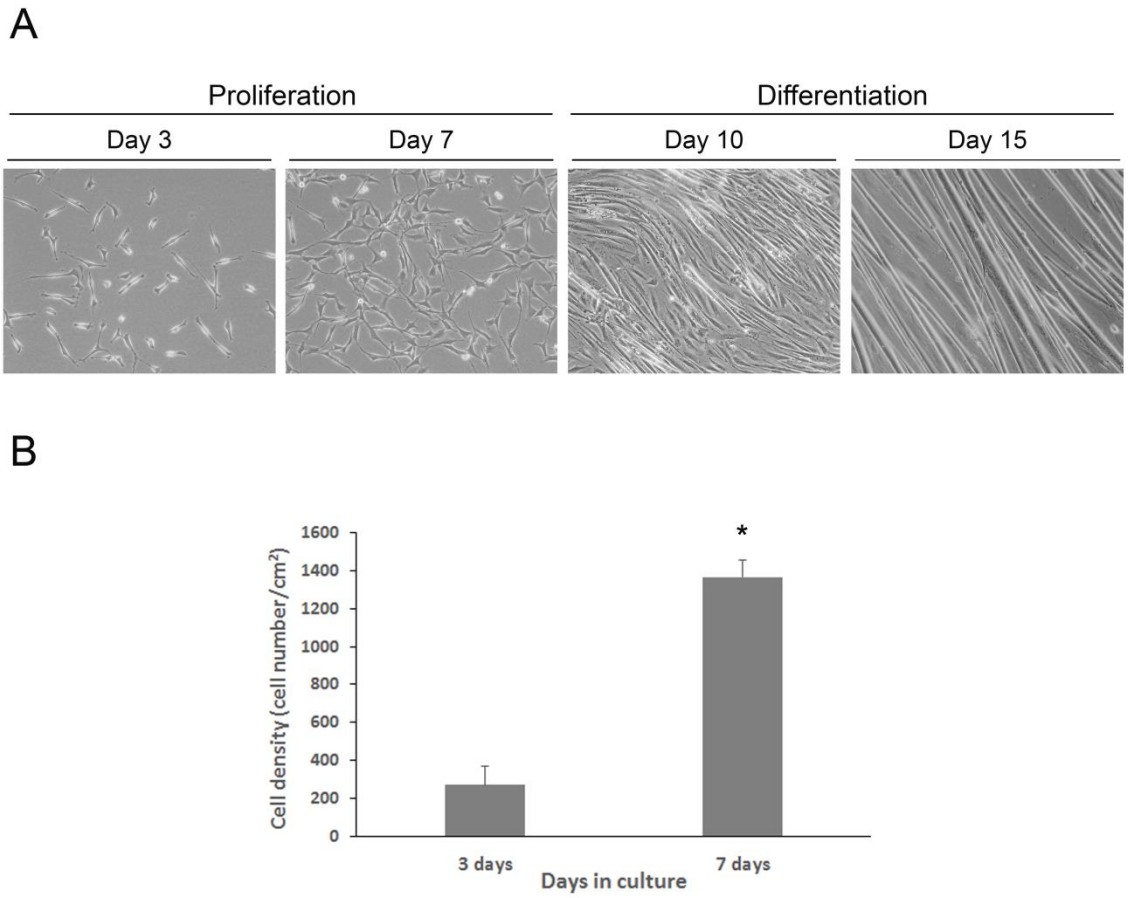

Figure 1. Myogenic proliferation and differentiation properties of primary cultured rat MPCs. MPCs were isolated by enzymatic digestion procedures from rat medial gastrocnemius muscle biopsy samples. (A) MPCs expanded in primary culture exhibited spindle-like shape and a strong proliferation and differentiation capacity. Cells were induced to proliferate by incubation in growth medium for 7 days until they reached 70-80% confluence, and then culture medium was changed to differentiation medium to induce the cells to differentiate for 7-8 more days. After inducing differentiation, MPCs demonstrated the capacity to fuse and form polinucleated myotubes with spontaneous contractile properties (see Supplementary video 2). Images show microphotographs at 100x of augmentation obtained under optical microscopy (Olympus IX71 microscope). (B) Cell number of primary cultured rat MPCs was measured by using a Neubauer counting chamber under optical microscopy (Olympus IX71 microscope).

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In vivo MPC transplantation into injured site of Medial Gastrocnemius muscle

Next, we induced a focal skeletal muscle injury to medial gastrocnemius muscle and the injured rats were divided into three groups: Control (Vehicle injection), MPC transplantation group (Cells), and the group receiving MPCs and treadmill running (Cells-Exercise). Before cell transplantation, proliferating MPCs were transfected with the retroviral expression vector pIRES2-DsRed2, which expresses red fluorescent protein, in order to localize cells after transplantation and to determine whether MPCs would participate in the formation of new muscle fibers *in vivo*. The efficiency of retroviral infection of MPCs was confirmed by the presence of red-fluorescence in transfected MPCs cells (Figure 2A). Red-labeled MPCs were successfully implanted 36 h after muscle injury by intramuscular US-guided injection into the injured area of medial gastrocnemius muscle (Supplementary video 1). 2 weeks after cell transplantation, a large number of red-fluorescent (+) muscle cells were detected at the site of the injury (Figure 2B) in transplanted muscles (Cells and Cells-Exercise groups), but not in control group. We could not detect transplanted cells outside of the injured area in injured skeletal muscle.

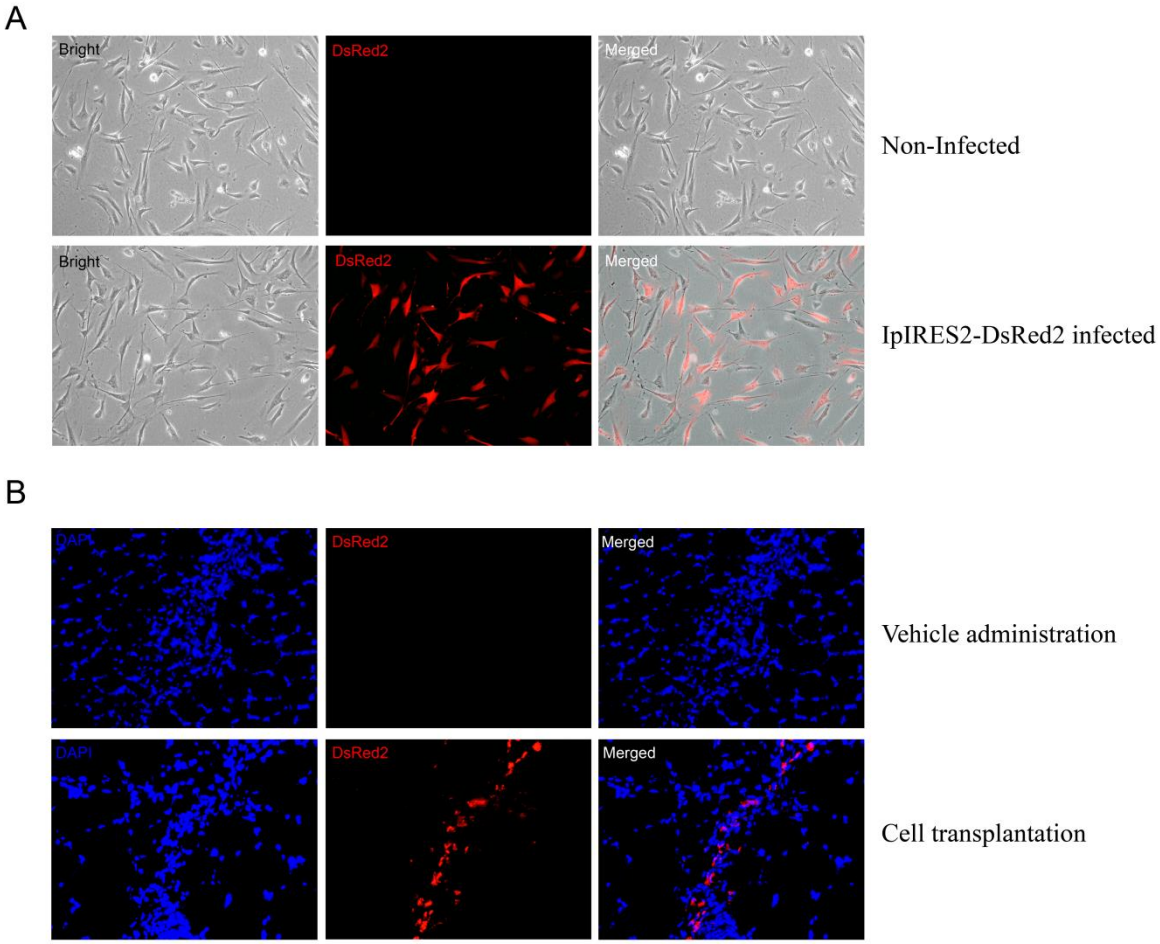

Figure 2. Localization of red-labelled MPCs before and after intramuscular transplantation. (A) Before intramuscular transplantation, proliferating MPCs were transfected with the retroviral vector pIRES2-DsRed2 and red-fluorescence in labelled MPCs was confirmed by fluorescence microscopy in cell cultures. **(B)** 2 weeks after cell transplantation by intramuscular US-guided injection into the injured area of medial gastrocnemius muscle, the red-fluorescence(+) labelled muscle cells were successfully detected in cross-sections of medial gastrocnemius transplanted muscles. Images show (x100) microphotographs obtained by using a BX-61 microscope (Olympus) equipped with a DP72 camera (Olympus) and CellSens® Digital Imaging software (version 1.9)

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335 **Effect of Treatments on Gastrocnemius Muscle Force**

336 The effect of the different treatments on muscle force recovery was determined by comparing
337 the maximum tetanus force (TetF) percentage of injured versus contralateral uninjured muscle
338 among the different groups of animals.

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340 The animals in the Vehicle group showed an approximate 22% decrease in TetF of the injured
341 versus healthy contralateral muscle: 78.2% ± 5,1% (P= 0.008) (Figure 3). Both treatments (Cells
342 and Cells-Exercise groups) produced a strong increase of TetF in the injured muscle with
343 respect to Vehicle group after 2 weeks of treatment (102.6% ± 4.0% for MPCs transplantation
344 group and 101.5% ± 8.5% for combination therapy-group, TetF of the injured vs healthy
345 contralateral muscle; P < 0.001 vs vehicle group).

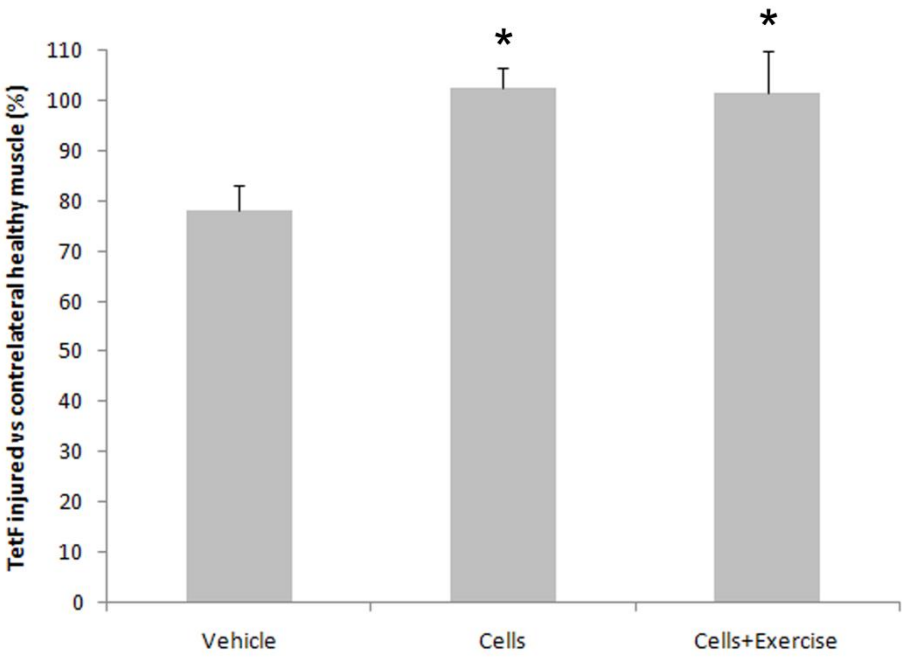

Figure 3. Measurement of gastrocnemius muscle force after treatments. Gastrocnemius muscle force was measured at the end of the treatments (14 days post-injury). Data represent maximum tetanus force (TetF) normalized to muscle mass for the injured vs contralateral control muscles. Values are presented as median ± SD. * P<0.01 vs vehicle-injected group.

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Analysis of muscle regeneration and fibrosis.

Next, we analyzed the cross-sectional area of the myofibers in the injured area after the treatment trial. A statistically significant increase in myofiber' CSA was found in rats belonging to Cells and Cells-Exercise groups when compared to vehicle-injected animals (247.5 ± 10.7 μm^2; $P < 0.001$, Figures 4A and B). Moreover, the Cells-Exercise group showed the largest increase in size (686.0 ± 11.6 μm^2) which was significantly higher than in Cells group (612.3 ± 21.4 μm^2; P= 0.001) demonstrating a synergistic effect of cell therapy and exercise treatments in the treatment of injured skeletal muscle.

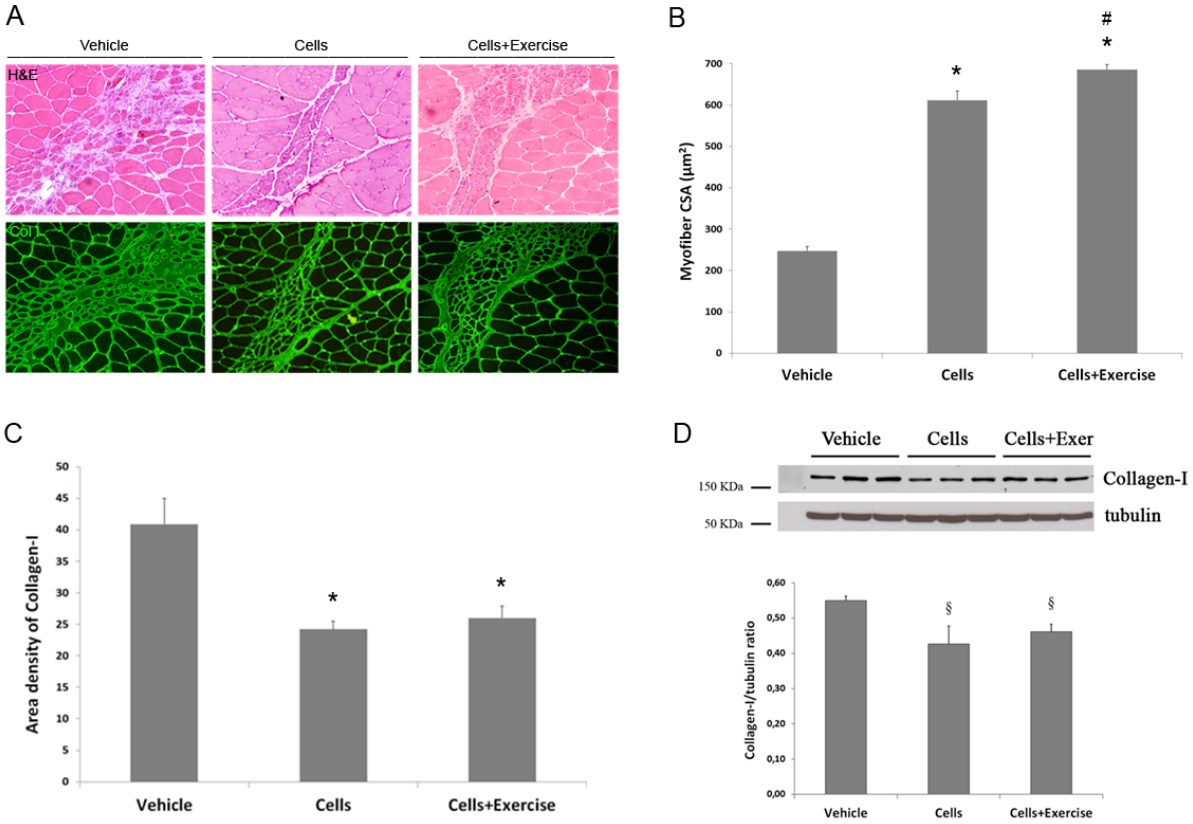

Figure 4. Analysis of the cross-sectional area of myofibres and degree of fibrosis in rat gastrocnemius muscle. (A) Representative images (N=8 for each treatment) show Hematoxylin-Eosin stained gastrocnemius muscle cross-sections (upper panel) and immunofluorescence analysis for collagen-I (lower panel) after treatments (14 days post-injury). Images show (x100) microphotographs for all panels. (B) Cross-sectional area (CSA) of newly formed muscles fibres was measured in collagen-I immunofluorescence microphotographs. Three images were randomly selected within the injured area of gastrocnemius muscle cross-sections by using a BX-61 microscope (Olympus) equipped with a DP72 camera (Olympus) and CellSens® Digital Imaging software (version 1.9). The CSA values of 200-300 fibres per muscle were calculated using Image J software version 1.46 (NIH, Bethesda, MD), based on a calibrated pixel-to-actual size (µm) ratio. Values are presented as median ± SEM. * P<0.001 vs vehicle-injected group, # P<0.05 vs Cells group. (C) The degree of fibrosis was evaluated in Collagen-I immunofluorescence images by determining the area of Collagen-I respecting to the total area in microphotographs. The percentage of collagen-I area was measured by using the Threshold Colour plugin for Image J software version 1.46 (NIH) as an average between 3 and 4 images in every muscle sample. The same threshold set was used for all samples analysed. Values are presented as median ± SD. * P<0.01 vs vehicle-injected group. (D) Collagen-I expression levels were corroborated by western blot analysis. § P<0.05 vs vehicle-injected group.

The degree of fibrosis was evaluated by the expression of collagen type I in muscle samples after 2 weeks of treatment (Figure 4, A and C). The Cells- and Cells+Exercise- groups showed

significantly less collagen type I deposition (24.2% ± 1.3% and 26.0% ± 1.9% of collagen type I positive area vs total area, respectively) than in vehicle group (40.9% ± 4.1% of collagen type I positive area vs. total area, $P < 0.001$). We demonstrated a strong reduction of intramuscular fibrosis by MPCs transplantation and combining it with exercise therapy (approximately 42% and 37% decrease in Cells and Cells+Exercise groups, respectively). Collagen type I levels were also analyzed by western blot analysis (Figure 4D) and we corroborated the decrease in collagen type I to be 22% ($P < 0.05$) and 16% ($P < 0.05$) in Cells and Cells-Exercise groups with respect to Vehicle group.

We then determined the presence of newly formed myofibers during the regeneration process by immunofluorescence analysis of dMHC expression levels at 2 weeks after treatments (Figure 5A). The participation of the intramuscularly injected MPCs in the formation of newly formed muscle fibers was corroborated by analyzing the co-localization of the red fluorescence coming from the injected MPCs and the expression of dMHC (labeled in green fluorescence) detected by immunofluorescence at the site of the muscle injury (Figure 5A). The co-localization of red (MPCs) and green (marker of newly forming myofibers) demonstrates that the injected MPCS are participating in the formation of new muscle fibers to repair the injured muscle segment. dMHC expression was quantified as the percentage of dMHC-positive area versus total muscle area in microphotographs (Figure 5B). We found a significant decrease in the number of dMHC-positive regenerating myofibers in Cells and Cells-Exercise groups (4.3% ± 2.6% and 4.1% ± 1.5%, respectively) than inVehicle group (14.8% ± 13.9%). The reduction in dMHC levels was 73% ($P < 0.001$) and 71% ($P < 0.001$) in Cells and Cells-Exercise groups. This demonstrates the accelerated replacement of the embryonic-developmental myosin isoform by mature muscle myosin isoforms in regenerating myofibers. No significant differences in dMHC levels were found between the Cells and Cells-Exercise animal groups. These data were also corroborated by western blot analysis (Figure 5C) where a reduction of 46% ($P < 0.05$) and 49% ($P < 0.05$) in dMHC levels was detected in Cells and Cells-Exercise groups with respect to Vehicle-injected animals.

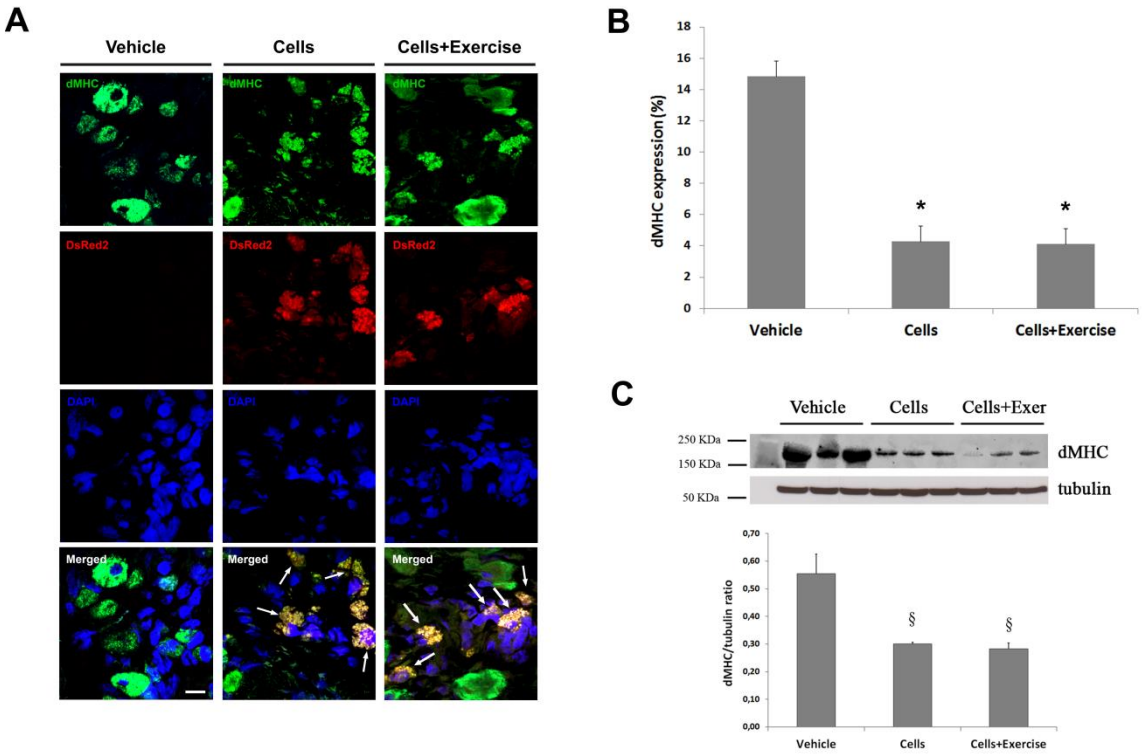

Figure 5. Analysis of dMHC expression levels in rat gastrocnemius muscle. (A) Representative images (N=8 for each treatment) show immunofluorescence analysis for dMHC after treatments (14 days post-injury) in gastrocnemius muscle cross-sections. The participation of transplanted MPCs in the formation of new muscle fibres was corroborated by the colocalization (white arrows) of the red fluorescence coming from MPCs with the myotube's marker dMHC analysed by immunofluorescence and labelled in green fluorescence. Images show (x100) microphotographs obtained by using a high resolution LSM 980 with Airyscan confocal microscope (Zeiss) and Zen 3.0 imaging software (version 3.0.79.00004). Scale bar indicates 10 µm. (B) dMHC expression levels were analysed by measuring the average green fluorescence signal intensity in dMHC immunofluorescence microphotographs obtained by using a BX-61 microscope (Olympus) equipped with a DP72 camera (Olympus) and CellSens® Digital Imaging software (version 1.9), and the signal intensity was determined by using the Image J software (version 1.46). The dMHC levels are presented as the percentage of dMHC-positive area respecting to the total area of the image. Values are presented as mean ± SD obtained from 3 to 4 images of every muscle sample. * P<0.001 vs vehicle-injected group. (C) dMHC expression levels were corroborated by western blot analysis. § P<0.05 vs vehicle-injected group.

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DISCUSSION

We have evaluated the individual effect of MPCs transplantation, and the combination of MPCs administration with early active rehabilitation protocol, to enhance muscle regeneration in an experimental rat skeletal muscle injury-model which closely reproduces skeletal muscle strain injuries seen in human athletes [13]. The advantages of our surgically-induced skeletal muscle injury animal model in rats include its capacity to reliably induce muscle injuries in rats in a quick, easy and highly reproducible manner, since it is based in the generation of a transversal biopsy procedure using identical commercially-available biopsy needles in all the animals for injury induction. Importantly, our muscle injury model in rats has not only demonstrated to follow the natural history of muscle regeneration after muscle strain injuries, showing the same highly orchestrated processes of necrosis, inflammation and regeneration observed in animal models of spontaneous skeletal muscle strain injury such as the dystrophic mdx-mice, but it has also demonstrated to imitate the muscle strain injuries observed in the human sports clinics by the MRI evaluation [13] when MRI images from the rat model were compared to MRI imaging data from muscle strain injuries in professional athletes [13].

MPCs have been postulated as a valuable source of stem cells for therapeutic purposes since they could be easily obtained from fresh muscle biopsies and isolated in primary cell culture by sequential enzymatic digestion and pre-plating techniques [11,19]. Although the clinical application of autologous MPCs transplantation has been already tested in several clinical trials for the treatment of Duchenne Muscular Dystrophy, sphincter-related urinary or fecal incontinence, and chronic heart failure diseases [9,18,21,23,51,54], only few preclinical studies have evaluated the potential benefits of intramuscular MPCs transplantation for the treatment of skeletal muscle injuries [4,27,34,39,42,53]. All these clinical applications highlight MPCs as a promising source of stem cells to develop new therapeutic strategies for the treatment of muscle-related pathologies.

Skeletal muscle biopsy samples could be easily harvested by means of simple minimally invasive procedures in an ambulatory clinic environment, providing easy access to muscle stem cells for their clinical use in musculoskeletal tissue regeneration. Cell culture methodology for isolating MPCs has been optimized based on the differential adherence of cells by pre-plating purification techniques [20,24]. This isolation procedure is based on their slow adhesion characteristics, which leads to the isolation of a purified cell population with a strong myogenic potential. Before using primary cultured MPCs for intramuscular injection into injured rat gastrocnemius muscle, we demonstrated their myogenic potential by performing *in vitro* differentiation analysis. The MPCs demonstrated *in vitro* capacity to fuse and form fully differentiated myofibers with spontaneous contractile properties (Supplementary video 2) which corroborates their regenerative potential when used in preclinical studies *in vivo*.

Our results confirmed the beneficial effect of MPCs-based therapy showing a complete recovery of the muscle force in all MPCs-treated animals after 14 days of cell transplantation. Since both groups of animals treated with cells (Cells and Cells+exercise groups) reached the maximum muscle force at 14 days after treatment, no differences between them were found in TetF determination. However, the improvement in muscle force was concomitant with the increase in the size of regenerating myofibers in cell-transplanted animals, since both Cells and Cells-Exercise groups showed 2.47- and 2.77-fold increase in myofibers' CSA when compared to vehicle-injected animals. More importantly, we found a synergistic effect of cell therapy when it was combined with the exercise-based rehabilitation protocol. The combination of both treatments promoted a significant increase of the size of regenerating myofibers (1.12-fold of increase in myofibers' CSA in Cells-Exercise group versus Cells group). Our data are in agreement with previously published data showing the synergistic effect of treadmill running exercise and intramuscular MPC transplantation to improve skeletal muscle healing in a model of muscle contusion injury in mice [4]. MPC-based cell therapy not only promoted the growth of regenerating muscle fibers and muscle force recovery but also accelerated the muscle regeneration process as demonstrated by the strong decrease in the expression levels of the

dMHC, a marker for primitive skeletal muscle fibers. All MPC-treated animals, both Cells and Cells-Exercise groups, revealed a robust decline (more than 70%) in the number of dMHC-positive regenerating myofibers when compared to Vehicle group. This finding indicates a faster replacement of the developmental/embryonic MHC isoform by adult MHC isoforms in regenerating muscle fibers and demonstrates the acceleration of muscle healing induced by intramuscular MPCs transplantation. In addition, MSDC-based therapy also induced a strong reduction of intramuscular fibrosis in both Cells and Cells-Exercise groups of animals, which showed approximately 40% of decrease of intramuscular collagen type I deposition in the site of the injury after 2 weeks of treatment. Our results demonstrate the effective anti-fibrotic effect of intramuscular MPCs administration in the prevention of intramuscular fibrosis and scarring caused by injury.

The enhanced muscle regeneration by MPC-therapy could be explained by the substantial benefits of the external addition of muscle precursor cells at the site of muscle injury during the first stages of muscle regeneration process. Skeletal muscle activates a self-repair process in response to injury that is divided in three highly synchronized overlapping phases [10,12,25,26,32]: inflammation (1-3 days post-injury), regeneration (encompassing up to 3 weeks post-injury and remodeling phases (3 weeks - 6 months post-injury). During the natural healing process, the dMHC-positive regenerating myofibers can be detected as soon as at 3 days post-injury, starting to appear in the periphery of the injured area [13]. This is in line with the activation of muscle precursor cells that begins within hours after damage. So called *committed* satellite cells differentiate into myoblast immediately [32,44,57] whereas *stem* satellite cells start to proliferate, then differentiate into myoblasts and then fuse together to form the newly regenerating muscle fibers[32,44,57]. It has been reported that the appearance of desmin-expressing myogenic cells can be detected as soon as after 12 h post-injury in adult rat skeletal muscle injury models [44,57]. Since the effectiveness of muscle regeneration process is probably directly related to the number of muscle progenitor cells and myoblasts present in the site of the injury, where they co-exist with inflammatory cells during the first stages of muscle healing process, the MPC-based

therapy by intramuscular transplantation increases the amount of myogenic cells present in the site of the injury and accelerates the formation of new myofibers and muscle regeneration. This is of utmost importance as the fundamental problem preventing the injured skeletal muscle achieving true regeneration is because the regenerating muscle fibers are very poor at extending to the intertwining scar tissue after they have filled the damaged basal lamina cylinders [1,26,32,56]. Thus, the scar tissue always develops between the ruptured myofibers in injured skeletal muscle [1,26,32,56]. The addition of exogenous muscle stem cells to the injured area could fill the rupture area with muscle tissue instead of fibroblast produced scar tissue that would accumulate between the ruptured muscle fibers without intervention [32]. In addition, taking into account that the activation of muscle stem cells occurs promptly after muscle damage (during the first days immediately after the injury), the optimal therapeutic time margin for intramuscular administration of MPCs could be framed between 24 h and 3 days post-injury, i.e. after the formation of preliminary fibrin-rich extracellular matrix, which works as a scaffold to retain the transplanted stem cells between the ruptured muscle fibers. Here, we have demonstrated the robust beneficial effects of the intramuscular transplantation of MPCs at 36 h post-injury, which promotes recovery of injured muscle both at functional and structural levels, showing a complete recovery of muscle force as well as the robust stimulation of regenerating myofibers' growth in the injured skeletal muscle treated with MPCs.

Our data demonstrates the *in vivo* synergistic effect of combining intramuscular administration of MPCs with post-injury exercise-based therapies to promote skeletal muscle healing. Post-injury exercise has already demonstrated beneficial effects on muscle healing both in humans [22,41,45,46] and animal models of skeletal muscle injury [2,4,14,33]. As previously reported in the surgically-induced muscle injury rat model, post-injury treadmill running provides substantial therapeutic effect when given alone, whereas no synergistic effect was detected when exercise-based treatment was combined with intramuscular Platelet Rich Plasma (PRP) injection [14]. Unlike the effect of PRP on muscle injuries, which does not add any beneficial effect to early, active rehabilitation protocols neither in animal models [14] nor in humans [22,45], MPC-based cell

therapy demonstrates a synergistic effect when combined to post-injury exercise to improve skeletal muscle healing in rats. It may be that the exercise provides the same "regenerative" stimuli for MPC as it provides for the native cells during the repair of injured skeletal muscle [31,32]. This may further enhance their biological potential in tissue regeneration.

A potential limitation of our study, although MPCs have demonstrated an important therapeutic effect on the treatment of muscle injuries, is that their therapeutic use would require to obtain them previously as a preventive measure for the application of cell therapy. This is because obtaining the MPCs requires a muscle biopsy followed by their expansion in the culture for several days. However, one could push back the injection of MPCs after the inflammatory period, i.e. after day 3, even though that we used 36 h delay in their administration. This scenario is supported by previous studies which have demonstrated that skeletal muscle regeneration does not progress from early myotube formation before the regenerating cells can use aerobic metabolism as their energy source [28,30]. As the angiogenesis-induced vascular supply that converts the metabolism of regenerating muscle cells to aerobic takes 5-7 days and the scar formation does not take place before that [28–30], the potential therapeutic window of stem cell therapies could be larger than anticipated. A late transplantation time-point could provide also benefits such as the improved survival of translanted stem cells as they would have less hypoxia to withstand and could have more adherence sites in early, loose granulation tissue that is about to be produced. Thus, more research is needed to demonstrate the ideal transplantation time-point of the MPCs in the treatment of skeletal muscle injury, but our positive results encourage for these future studies. Additionally, regarding the potential transfer of MPC-based cell therapy to human clinics, based on the results obtained in our rat model and considering the size of muscle lesions commonly observed in athletes, we estimate that for the transfer of the therapeutic protocol and escalation to humans, approximately 20-25 million cells would be needed for intramuscular administration at the site of the injury. Despite the fact that obtaining tens of millions of cells is very affordable in terms of cell culture and expansion, further research is needed to establish and optimize the human therapeutic protocol and the precise

number of cells to be intramuscularly administered to promote optimal muscle healing and reduced recovery time in athletes.

In summary, we have addressed the beneficial effects of intramuscular MPC transplantation and the potential interaction with early active rehabilitation after injury in a well characterized experimental skeletal muscle injury-model that closely mimics injuries seen in human athletes. Our results show that the ultrasound-guided intramuscular MPC transplantation in the site of the injury at 36 h after skeletal muscle lesion significantly promoted muscle regeneration, decreased fibrosis formation within the injured muscle and accelerated the functional recovery of the muscle after injury. Our study also demonstrates the synergistic effect of both MPC-based therapy and early active rehabilitation-protocol to enhance the repair of the injured skeletal muscle when these two therapies are combined.

CONCLUSION

Our results demonstrate beneficial effects of early active rehabilitation-protocol or single intramuscular MPC-injection on muscle recovery in a rat injury-model which resembles the sports-related muscle lesions in athletes. We show here that MPC transplantation has a strong beneficial effect on muscle healing in a surgically-induced muscle injury in rats. In addition, we have demonstrated *in vivo* the synergistic effect of the combination of both MPC-based therapy and early active rehabilitation-protocol to stimulate muscle healing after traumatic muscle injury. The fact that cell therapy and exercise showed beneficial synergistic effects points to future therapeutic strategies to accelerate muscle healing after injury in athletes subjected to MPC transplantation and post-injection rehabilitation protocols based on early, active mobilization.

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