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A “noisy” electrical stimulation protocol favors muscle regeneration *in vitro* through release of endogenous ATP

Alessandra Bosutti^a, Annalisa Bernareggi^a, Gabriele Massaria^{a,b}, Paola D’Andrea^a, Giuliano Taccola^{c,d}, Paola Lorenzon^a and Marina Sciancalepore^a

^aDepartment of Life Sciences and Centre for Neuroscience B.R.A.I.N., University of Trieste, Via A. Fleming 22, I-34127 Trieste, Italy

^bArea Science Park, Padriciano, 99, I-34149 Trieste, Italy

^cDepartment of Neuroscience, SISSA, Via Bonomea 265, 34136 Trieste, Italy

^dSPINAL (Spinal Person Injury Neurorehabilitation Applied Laboratory), Istituto di Medicina Fisica e Riabilitazione (IMFR), via Gervasutta 48, 33100 Udine, Italy.

Short title: EMG*stim* favors muscle regeneration *in vitro*

Correspondence to: Marina Sciancalepore, PhD

Department of Life Sciences, University of Trieste, Via Fleming, 22, 34127 Trieste, Italy

Email: msciancalepore@units.it

Tel. +390405588613

ABSTRACT

An *in vitro* system of electrical stimulation was used to explore whether an innovative “noisy” stimulation protocol derived from human electromyographic recordings (EMG*stim*) could promote muscle regeneration. EMG*stim* was delivered to cultured mouse myofibers isolated from *Flexor Digitorum Brevis*, preserving their satellite cells. In response to EMG*stim*, immunostaining for the myogenic regulatory factor myogenin, revealed an increased percentage of elongated myogenin-positive cells surrounding the myofibers. Conditioned medium collected from EMG*stim*-treated cell cultures, promoted satellite cells differentiation in unstimulated myofiber cell cultures, suggesting that extracellular soluble factors could mediate the process. Interestingly, the myogenic effect of EMG*stim* was mimicked by exogenously applied ATP (0.1 μ M), reduced by the ATP diphosphohydrolase apyrase and prevented by blocking endogenous ATP release with carbenoxolone.

In conclusion, our results show that “noisy” electrical stimulations favor muscle progenitor cell differentiation most likely via the release of endogenous ATP from contracting myofibres. Our data also suggest that “noisy” stimulation protocols could be potentially more efficient than regular stimulations to promote *in vivo* muscle regeneration after traumatic injury or in neuropathological diseases.

Keywords: Differentiation, Electrical stimulation, Skeletal muscle, Myofibers, ATP, Satellite cells.

Abbreviations:

AU	Arbitrary Units
AUC	Area Under the Curve
CBX	Carbenoxolone
CM	Conditioned Medium
DAPI	4',6-diamidino-2-phenylindole
EMG <i>stim</i>	Electromyographic stimulation
ES	Electrical Stimulation
FDB	Flexor Digitorum Brevis
SC	Satellite Cell

Introduction

Direct electrical stimulation (ES) of muscles has been demonstrated to mimic nerve activity and therefore has been proposed as a tool to counteract muscle atrophy and to enhance muscle strength in patients suffering from chronic debilitating conditions. Benefits depend on the efficacy of the stimulation patterns and the most appropriate stimulation parameters are still under debate [1,2]. We have already demonstrated that a “noisy” ES protocol derived from human electromyographic recordings, named *EMGstim*, is capable of eliciting *in vitro* mouse myotube contractions more efficiently than “regular” conventional stimulation protocols. More specifically, we observed that in cultured myotubes, *EMGstim*, characterized by biphasic voltage pulses of various duration, amplitude and frequency, was able to elicit action potentials and Ca^{2+} release from the sarcoplasmic reticulum of cultured myotubes *in vitro*, at a lower stimulus strength than regular 1 Hz, 1 ms pulse stimulations. These results suggest that *EMGstim*, as a “noisy” protocol, could be potentially more effective in eliciting contractile activity in human patients, thereby limiting the common occurrence of pain and fatigue during ES [3]. Although the effect of ES protocols has been quite extensively studied at the level of skeletal muscle fibers [4-6], the effects on the regenerative response of satellite cells (SCs), the resident myogenic precursors, still remains unknown. SCs within adult skeletal muscle represent a heterogeneous population of undifferentiated mononuclear cells, located under the basal lamina of each myofiber [7]. Their number depends on the species, age and muscle fiber type. Even if they constitute around 5% of the muscle nuclei in the adult mice fiber [8], SCs are important for the maintenance and regeneration of skeletal muscle. Activation of SCs by external stimuli like exercise or injury induces cell proliferation and differentiation, recapitulating the embryonic and fetal program of muscle differentiation.

Satellite cell activation, proliferation and differentiation can be studied in isolated and cultured mouse FDB myofibers [9,10]. After muscle cell plating, SCs detach from the myofibers, divide, migrate and fuse into myotubes in 4-6 days. The different phases of SC myogenesis can be identified by evaluating the expression of specific transcription factors [11].

In the present work, *EMGstim* was delivered to isolated mouse FDB myofibers along with their resident SCs. Immunolabeling for Pax7 and myogenin were then carried out to monitor the effect of *EMGstim* on SC number and differentiation, respectively.

Our results show that, in the absence of motor and sensory innervation, the muscle cell activity promoted by *EMGstim* favors myogenic progenitor differentiation. A molecular mechanism based on the release of endogenous ATP from myofibers during the *EMGstim*-induced contractile activity is proposed.

Materials and methods

FDB myofiber culture

Skeletal mouse muscle fibers with the resident SCs were obtained from the dissociation of *Flexor Digitorum Brevis* (FDB) muscles of 6 to 8-weeks old C57BL/6J male mice [12,13].

For muscle dissection, mice were anesthetized and sacrificed by cervical dislocation as approved by local Animal Care Committee and in agreement with the European legislation (2010/63/EU). FDB myofibers were isolated from both hind feet muscles of a single mouse for each preparation. Briefly, FDB muscles were enzymatically treated for 1 h in ice and 1 h at 37 °C with Type I collagenase 0.3 % (wt/v), in Tyrode's solution supplemented with Fetal Bovine Serum (10%), penicillin (100 Units/ml) and streptomycin (100 µg/ml). Single fibers (750-900 for each mouse) were isolated by mechanical dissociation with Pasteur pipettes with decreasing tip diameters and allowed to settle on matrigel-coated (1 mg/ml) glass coverslips accommodated in 35-mm Petri dishes. Cultures were covered with DMEM high glucose enriched with Horse Serum (5%), L-Glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Dishes were maintained in an incubator at 37°C in saturated humidity and in CO₂ (5%)-enriched air. The medium was replaced every 48 h.

Assessment of cell contractions

Myofiber twitching was visualized using an inverted phase-contrast microscope (Axiovert S100, Carl Zeiss, Jena, Germany) equipped with a 40x objective. Images were collected by a digital camera (Sony Alpha 6300) at a sampling frequency of 100 Hz.

Immunofluorescence staining

FDB myofibers seeded on coverslips were fixed with a solution of 4% (w/v) paraformaldehyde in PBS for 20 min at 4 °C, followed by washing three times with PBS (10 min each). Cell permeabilization and the blocking of non-specific protein binding were conducted by incubation with 5% normal goat serum in PBS/0.1% Triton-X100 for 30 min. The staining was performed by incubation with a primary mouse monoclonal anti-Pax7 (1:8 dilution) or a mouse monoclonal anti-myogenin (1:20 dilution), overnight in normal goat serum at 4 °C. After three washes of 10 min with PBS/0.1% Triton-X100, coverslips were incubated with the secondary antibodies for 1.5 h at 4 °C (1:50, Alexa Fluor 594-conjugated Affine Pure goat anti-mouse IgG for anti-Pax7 staining or 1:100, Alexa Fluor 488-conjugated Affine Pure goat anti-mouse IgG for anti-myogenin staining). Nuclei were counterstained by

4',6-diamidino-2-phenylindole (DAPI,1:50). Finally, cells were washed three times with PBS/0.1% Triton-X100 and then mounted onto slides and visualized under a Leica DMLS fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Images were captured with a Leica DC300F camera, coupled to a Leica LM50 acquisition software. Image analysis, measurements of cell morphology and nuclei counting were performed by ImageJ-NIH software. Image sizing, cropping and overlays were obtained with Adobe Photoshop CC (Adobe Systems Incorporated, San Jose, CA). Only dishes containing a comparable number of myofibers (around 100-150) were used for immunofluorescence detection. In the count, we considered the SCs associated to the fibers and those that migrate from them. The aspect ratio (ratio between main and minor axis) of single cells was measured in bright field microscopy in cells expressing myogenin positive nuclei.

Each set of experiments was carried out on three independent cell culture preparations. At least 30 different optical fields were examined in each coverslip corresponding to a minimum myogenic cell number equal to 300.

Electrical stimulation

ES was carried out on FDB myofibers 20 h after seeding, when SCs were still adhered to the fibers. Coverslips with isolated myofibers were mounted on the stage of an inverted Axiovert microscope (Carl Zeiss, Germany) and stimulated in a 35 mm Petri dish by a custom-built device. Field ES was delivered by the programmable stimulator STG 4002 (Multi Channel Systems, Reutlingen, Germany) connected to a couple of parallel platinum-iridium electrodes (0.2 mm in diameter), placed 1 cm apart and positioned 1-2 mm over the cells. Unless otherwise specified, the maximum voltage strength used was that capable of eliciting twitching in 60% of muscle fibers. EMGstim or bipolar 1 Hz, 1 ms pulses were always delivered in fresh DMEM. Cell cultures were maintained at 37 °C in 5 % CO₂ until they were processed for immunofluorescence.

For Ca²⁺ imaging experiments, each single myofiber was electrically stimulated with focal field stimulations using two concentric bipolar platinum electrodes (0.2 mm in diameter) positioned within 3 mm from each other. The inner pole was inserted in a glass pipette surrounded by the outer pole arranged as a wire coil. The concentric electrodes were positioned 1-2 mm over the considered myofiber.

Ca²⁺ imaging

Intracellular calcium concentration $[Ca^{2+}]_i$ was monitored using the fluorescent Ca^{2+} indicator fura-2 pentacetoxymethyl ester (Fura-2 AM). Ca^{2+} imaging experiments were performed on skeletal myofibers plated on matrigel-coated coverslips 20 h after seeding. Cell loading was carried out at room temperature, in dark condition, in DMEM plus 5 μ M Fura-2 AM. After 30 min, the loading solution was removed and the cells washed and bathed in DMEM for 15 min to allow complete de-esterification of the dye. Cells were visualized with a Zeiss Axiovert S100TV inverted microscope (Carl Zeiss, Jena, Germany) using an oil immersion 40x objective. Ca^{2+} measurements were carried out at room temperature exciting alternately, the cells at 340 and 380 nm, selected by a monochromator device equipped with integrated light source (Polychrome IV, Till Photonics, Gräfelfing, Germany). Fluorescence signals were collected by a CCD camera (SensiCam; PCO Computer Optics, Kelheim, Germany) at a sampling rate of 6.45 Hz. The monochromator and CCD camera were controlled by a software package (TILLvision, Till Photonics) also used for image processing. The ratio of fluorescence images (340/380) and the corresponding temporal plots representing the mean value of the fluorescence signal in regions of interest, were calculated off-line.

Ca^{2+} imaging experiments were carried out on a single cell culture preparation. A total of 8 myofibers were considered for the analysis; area and peaks of at least 40 Ca^{2+} transients for each cell were averaged.

Chemicals

L-Glutamine, penicillin and streptomycin were purchased from Euroclone (Milano, Italy); Fetal Bovine Serum from Gibco (Burlington, ON, Canada); Matrigel from Corning (Tewksbury, MA, USA). Mouse monoclonal anti-Pax7 MAB1675 was from R&D Systems (Minneapolis, MN, USA); mouse monoclonal anti-myogenin (5FD), sc-52903 from Santa Cruz Biotechnology (Dallas, TX, USA). Alexa Fluor 594-conjugated Affine Pure goat anti-mouse IgG and Alexa Fluor 488-conjugated Affine Pure goat anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All the other chemicals, unless otherwise stated, were from Sigma (St. Louis, MA, USA).

Statistical analysis

Data were analyzed with GraphPad Prism 4.00 (GraphPad Software, San Diego, CA, USA). A normality *test* was used to determine whether sample data were drawn from a Gaussian distributed population. For parametric data, statistical significance was determined using *t*-

test or one-way ANOVA analysis followed by the Tukey's multiple comparison test. Non-parametric data were analysed by Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. Unless otherwise stated, results were expressed as mean \pm standard error (SEM). A p value of <0.05 was considered statistically significant.

Results

EMGstim elicited $[Ca^{2+}]_i$ transients and contractions in FDB myofibers

Ca^{2+} transients elicited in response to focal ES were measured in single FDB myofibers loaded with the Ca^{2+} indicator Fura-2AM. The “noisy” protocol *EMGstim*, variable in amplitude, frequency and pulse duration [3], Fig. 1a), was able to elicit intracellular Ca^{2+} transients and contractions at around 1 Hz as well as the standard protocol of ES at 1 Hz of stereotyped 1 ms pulses (Fig. 1b). To compare the ES-elicited Ca^{2+} transients, the two protocols were applied to each imaged myofiber at the same maximum strength (mean peak-to-peak amplitude 3.45 ± 0.17 V, $n = 8$). The area under the curve (AUC), relative to the baseline, and the peak amplitude were measured and averaged. Interestingly, *EMGstim* elicited Ca^{2+} transients with significant larger area and higher amplitude ($p < 0.05$) than 1 Hz stimulation protocol (Fig. 1c). In the same cells, the mean Ca^{2+} AUC induced by *EMGstim* and 1 Hz ES were 2438.00 ± 288.80 AU ms and 1434.00 ± 146.70 AU ms, respectively. The mean Ca^{2+} peak amplitude induced by *EMGstim* was 10.47 ± 1.44 Arbitrary Units (AU) whereas it was 6.58 ± 0.72 AU ($n = 8$ myofibers) at 1 Hz ES.

EMGstim did not affect the number of myogenic precursor cells

Immunolabelling for the marker of quiescent and activated SCs Pax7, performed 2 h after FDB cell plating, revealed the presence of SCs adherent to the single myofibers (1-2 for each of them, Fig. 2). SCs proliferate then as myoblasts [14,15] and migrate far from the myofibers forming new myotubes during the time in culture. In our experimental conditions, migration occurred 36 h after seeding. To determine the effect of *EMGstim* on the number of myogenic precursors, FDB myofibers were stimulated 20 h after seeding and Pax7 positive cells were then detected 36, 48 and 72 h after plating, in stimulated and unstimulated (control) cultures (Fig. 3a). At 36 and 48 h, the percentage of Pax7 positive nuclei per area in *EMGstim*-stimulated cultures did not differ from the controls (36 h: $63.75 \pm 3.70\%$ vs $62.09 \pm 4.17\%$; 48 h: $68.47 \pm 4.17\%$ vs $61.94 \pm 3.13\%$; Fig. 3b). In addition, 72 h after seeding, the percentage of Pax7-expressing nuclei per area tended to decrease in both stimulated ($45.65 \pm 4.20\%$) and controls ($50.08 \pm 5.86\%$; Fig. 3b). The latter observation was in line with the increasing number of cells committed to terminal differentiation reported around 72 h in culture [13], when Pax7 is down-regulated and the expression of the transcription factor myogenin increases.

EMGstim increased the proportion of myogenin-positive cells

72 h after FDB myofibre plating, SCs differentiation was determined by counting the percentage of myogenin-positive cells. Compared with the controls, EMGstim delivered for 1 h at 12 V maximum peak-to-peak voltage, increased the proportion of myogenin-positive nuclei ($44.70 \pm 0.84\%$ vs $36.76 \pm 0.80\%$, Fig. 4a, b). Furthermore, in EMGstim the prevalence of myogenic cells with an elongated morphology increased comparing with the controls, as shown by the increase in the aspect ratio ($5.12 \pm 0.17 \mu\text{m}$ vs $3.96 \pm 0.16 \mu\text{m}$; Fig. 4 c, d).

Regular 1 Hz and 10 Hz, 1 ms ES protocols were delivered to the cultures at 24 V, the output voltage able to induce 60 % of myofiber contractions. 1 Hz, 1 ms ES protocol induced no significant changes in the proportion of myogenin-positive nuclei with respect to control cultures, even if the ES was delivered at a double strength (24 V, $42.81 \pm 1.41\%$ vs $39.10 \pm 1.43\%$, control).

In a different set of experiments 10 Hz, 1 ms regular pulses were elicited at 24 V inducing significant change in the proportion of myogenin-positive nuclei with respect to control cultures ($55.63 \pm 1.41\%$ vs $43.88 \pm 1.22\%$, control, $p < 0.001$). In the same cultures, compared with the controls, EMGstim delivered for 1 h at 12 V maximum peak-to-peak voltage, increased the proportion of myogenin-positive nuclei ($60.86 \pm 1.46\%$, $p < 0.001$), significantly different ($p < 0.05$) from what observed at 10 Hz. The prevalence of myogenic cells with an elongated morphology at 10 Hz was significantly different from the controls (aspect ratio $6.93 \pm 0.34 \mu\text{m}$ vs $5.00 \pm 0.21 \mu\text{m}$, $p < 0.001$) but not from EMGstim (aspect ratio 7.83 ± 0.30 , $p > 0.05$).

Soluble factors mediated the effect of EMGstim on myogenic differentiation

In an additional set of experiments, we tested the possibility that the stimulatory effects of EMGstim were due to the release of soluble factor (s) from skeletal myofibers. To this end, immunostaining for myogenin was performed in FDB myofibers in four different experimental conditions: i) controls; ii) 1 h stimulation with EMGstim, started 20 h after cell seeding; iii) unstimulated FDB myofibres cultured in the medium collected from another culture immediately after EMGstim-treatment (conditioned medium, CM); iv) EMGstim delivered as in (ii) but immediately followed by washout with fresh medium (replaced medium, RM). Immunostaining was performed 72 h after plating.

The exposure to CM partially mimicked the effect of *EMGstim* on cell differentiation. The percentage of myogenin-positive muscle progenitors in cultures exposed to CM ($46.57 \pm 1.62 \%$), was significantly higher than controls ($39.06 \pm 1.50 \%$) but remained lower than in *EMGstim* cultures ($57.25 \pm 1.89 \%$; Fig. 5a). In addition, CM, similarly to *EMGstim*, induced elongation of myogenin-positive cells (aspect ratio: $6.88 \pm 0.35 \mu\text{m}$; Fig. 5b). Collectively, the exposure to CM partially mimicked the effect of *EMGstim* on muscle cell differentiation. Moreover, in the RM cultures, both percentages of myogenin-positive cells ($40.29 \pm 1.33\%$ vs $39.06 \pm 1.50\%$) and the cell aspect ratio (4.18 ± 0.23 vs 4.91 ± 0.29) were similar to controls (Fig. 5a, b).

Taking into account all these observations, the results suggested a role for soluble factors released from myofibers in determining the stimulatory effects of *EMGstim* on SCs.

ATP as a potential soluble mediator for EMGstim-induced effect on myogenic differentiation

Extracellular ATP is released from contracting skeletal muscle fibers during ES [4,6] and promote SC activation [16,17]. This evidence prompted us to study if ATP released from ES/contracting fibers could be implicated in the favoring effect of *EMGstim* on myogenic differentiation. To test this hypothesis, we firstly analyzed the effect of exogenously-applied ATP on muscle cell differentiation. In particular, 20 h after seeding, increasing concentrations (0.01, 0.1 and $1 \mu\text{M}$) of ATP were added to the FDB cell cultures, incubated for a further 48 h (up to 72 h after seeding). At this time, the percentage of myogenin-positive cells was determined and compared to controls. Results showed that $0.1 \mu\text{M}$ ATP increased the percentage of myogenin-positive cells ($58.55 \pm 1.13\%$; Fig. 6).

The ATP diphosphohydrolase apyrase is an enzyme catalyzing the hydrolysis of ATP to its unphosphorylated catabolites. To test the involvement of ATP in mediating the *EMGstim* effect, cells were incubated in with apyrase (10 U/ml) 30 min before delivering the ES and during *EMGstim*. The presence of apyrase prevented both the increase in the number and the elongation of myogenic-positive cells induced by either *EMGstim* treatment and by CM (Fig. 7a, b). To further confirm the role of ATP, the connexin and pannexin 1 inhibitor carbenoxolone (CBX) was used to inhibit ATP release [18,19]. Preincubation in CBX ($5 \mu\text{M}$) prevented the *EMGstim* effect both on the percentage of myogenin-positive cells (Fig. 7 c) and cell morphology (Fig. 7d).

Figures

Fig. 1 $[Ca^{2+}]$ transients elicited by EMGstim and regular 1 Hz pulses. Representative intracellular $[Ca^{2+}]$ transients detected in the same cultured myofiber, during EMGstim (**a**) or 1 Hz pulses (**b**) elicited at 3 V (focal ES). On the top of each Figure, the stimulation protocol is shown. The fluorescence 340/380 ratio values are in Arbitrary Units. Scale bars, 5 s. The the mean areas under the curve (AUCs) and the mean Ca^{2+} peak amplitudes induced by EMGstim and 1 Hz ES, are represented (**c**, n= 8 myofibers). * $p<0.05$, ** $p<0.01$.

Fig. 2 Distribution of Pax7 positive cells in freshly isolated FDB myofibers. Representative merged image of Pax7 immunolabeled SCs (red) with DAPI nuclear counterstain (blue), 2 h after FDB myofiber seeding. Scale bar, 50 μm .

Fig. 3 EMGstim effect on Pax7 positive cells. **a** Merged images of Pax7 positive cells 36, 48 and 72 h after seeding in control conditions and after 1 h EMGstim. **b** Proportion (%) of Pax7-positive cells at 36, 48 and 72 h in culture in controls and after EMGstim. Scale bars, 100 μm .

Fig. 4 EMGstim effect on myogenin-positive cells. **a** Representative immunofluorescence-labeled myogenin-positive cells (green), DAPI counterstained nuclei (blue) and corresponding merged micrographs, at 72 h of seeding, in control conditions and in EMGstim treated cultures. Scale bars, 100 μm . **b** Percentage of myogenin-positive cells in control and EMGstim treated cultures. **c** Representative immunofluorescence-labeled myogenin-positive cells (green) merged with the same images captured in bright field microscopy in control and EMGstim treated cultures. Scale bars, 20 μm . **d** The aspect ratio of myogenin positive-cells in control and in EMGstim treated cultures. *** $p<0.001$ vs controls.

Fig. 5 Soluble factors mediated the EMGstim effect. **a** Percentage of myogenin-positive cells 72 h post seeding in control conditions, in cultures that underwent EMGstim (EMGstim), in cultures exposed to conditioned medium collected from EMGstim-treated cells (CM) and in cultures in which the medium was replaced immediately after stimulation (RM). See the text

for further details. **b** Quantification of the myogenin-positive cell morphology in *EMGstim*, CM and RM cultures. *** $p < 0.001$, ** $p < 0.01$ vs controls; §§ $p < 0.01$, §§§ $p < 0.001$ vs *EMGstim*.

Fig. 6 Exogenous ATP facilitated myogenic differentiation. Percentage of myogenin-positive cells in cell cultures treated with 0.01, 0.1 and 1 μ M ATP. ** $p < 0.01$ vs controls.

Fig. 7 Dephosphorylation of ATP and blockage of ATP release prevented the *EMGstim* effects. **a** Percentage of myogenin-positive cells (expressed as ratio on controls) in cultures stimulated with *EMGstim* (*EMGstim*), stimulated with *EMGstim* in the presence of apyrase (10 U/mL; AP+*EMGstim*) or cultured in CM from *EMGstim* treated cultures to which apyrase was added (AP+CM). **b** Morphology of myogenin-positive cells in the experimental conditions as in a. **c** Percentage of myogenin-positive cells (expressed as ratio on controls) in cultures stimulated with *EMGstim* (*EMGstim*) or stimulated with *EMGstim* in the presence of carbenoxolone (5 μ M; CBX+*EMGstim*). **d** Morphology of myogenin-positive cells in the experimental conditions as in c. § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ vs *EMGstim*.

Discussion

ES is recognized as a strategy for providing rehabilitation for individuals suffering from muscle atrophy, especially when physical therapy is limited. Mimicking nerve activity, it not only induces contractions but also drives gene expression patterns, activation of metabolic pathways [20-24] and SC-mediated muscle regeneration [25-27]. Intensive ES, activating a higher number of motor units, could likely be more efficient in inducing muscle potentiation but is often associated with pain [28,29] and muscle fatigue [30,31]. The optimization of the most appropriate parameters for ES thus remains under debate and investigation [25,2].

In the present work, we have studied the effect of the “noisy” stimulation pattern, *EMGstim*, on SC behavior. Compared with a regular stimulation pattern, the “noisy” protocol was already proved to be more effective in inducing firing, $[Ca^{2+}]_i$ changes and contractions in cultured mouse myotubes [3]. Here, we have shown that the “noisy” stimulation pattern *EMGstim* elicits higher Ca^{2+} transients in adult mouse FDB myofibers and favors the differentiation of their associated SCs. Moreover, our results indicate that endogenous ATP released by FDB myofibers during the contractile activity could be a crucial factor in enhancing the differentiation of the myogenic precursors.

The “noisy” stimulation pattern *EMGstim* was obtained from human electromyographic recordings and originally designed for the optimal recruitment of neuronal spinal networks [32-33]. In the present work, we provide evidence that in isolated adult FDB myofibers *EMGstim*, delivered at the same maximum strength of regular 1 Hz stimulations, is able to induce significantly higher increases in $[Ca^{2+}]_i$. *EMGstim* is characterized by a temporal summation of stochastic pulses with rest intervals of around 1 second. Most likely, the augmented Ca^{2+} signals induced by *EMGstim* depends on the features of the number of pulses characterizing *EMGstim* signals continuously variable in amplitude, duration and frequency [5]. The intrinsic variability characterizing the “noisy” ES, might facilitate a cumulative membrane depolarization responsible for the Ca^{2+} release from the sarcoplasmic reticulum during the excitation-contraction coupling, resulting in Ca^{2+} transients with bigger peaks and area.

Using the isolated FDB myofibers with their resident SCs, we also investigated the effect of *EMGstim* on muscle progenitors since the early phases of activation, proliferation and differentiation. We firstly observed that *EMGstim* did not alter the number of Pax7 positive cells indicating that it did not affect the survival and/or the proliferation of the myogenic precursors. Secondly, we noticed that the “noisy” waveform *EMGstim*, increased the number

of myogenin-positive cells, a phenomenon not observed in response to 1 Hz ES, even if delivered at a double voltage strength. Moreover, the elongated shape of the myogenin-positive cells, with respect to controls, highlights the favoring effect of *EMGstim* on myogenic differentiation. Since the ATP release in FDB fibers was found to depend on ES frequency, 10 Hz regular ES was tested, at which such release was found to be optimal [6]. A slight but significant higher effect of *EMGstim* on increasing the myogenin-positive cells was found, comparing with 10 Hz ES. The fact that *EMGstim* could be elicited at half maximum voltage strengthens the efficacy of such “noisy” ES protocol in inducing cell differentiation.

We also observed that CM, collected from *EMGstim*-treated FDB myofibers, fairly reproduced the effect of *EMGstim* on SCs. In particular, cell exposure to CM mimicked the effect on the elongation of myogenin-positive cells and increased the percentage of myogenin-positive cells, albeit less than *EMGstim*. From these data emerges the contribution of extracellular soluble factors released by the skeletal muscle fibers during ES stimulation. The lower efficacy of CM on SC differentiation could be attributable to the degradation of key signaling molecules mediating the *EMGstim* effect on muscle cell differentiation.

Exercise [34-35] as well as ES *in vivo* [36-37] have been associated with the secretion of ATP from skeletal muscle fibers, that not only circulates and attracts monocytes [38] but can also exert an autocrine/paracrine effect, easily unveiled in a controlled system such as C2C12 cell lines [17] or isolated myofibers [4]. Our observation that exogenous ATP mimicked the *EMGstim* effect on the number and morphology of myogenin-positive cells migrating from adult FDB myofibers suggested that the nucleotide could represent one of the key signaling molecules.

It is not excluded that other nucleotides such as guanosine 5' triphosphate might enhance muscle cell differentiation [39,40].

Extracellular ATP, released after muscle fiber contraction, has been already proposed to act autocrinally as a mediator of the excitation-transcription mechanism in skeletal muscle cells [4,41,6]. Our observations indicate that ATP could also act paracrinely on SCs. The finding that apyrase inhibited the effect of *EMGstim* and CM, rules out the involvement of ATP catabolites such as adenosine. As observed by others [4,42], also in our experimental conditions, ATP release was inhibited by CBX, suggesting that pannexins could mediate a regulated ATP efflux from skeletal myofibers. ATP is supposed to mediate different effects during myogenesis via the activation of purinergic receptors. In mouse SCs [43] and C2C12 myoblasts [17], ATP has been reported to activate ionotropic P2X receptors inhibiting

proliferation and inducing differentiation. ATP application in primary mouse cultures increased the expression of markers of muscle differentiation (myogenin, the cell cycle regulator p21 and myosin heavy chain) via the MAPK signaling cascade [43]. In C2C12 extracellular ATP controlled the levels of myogenin and the number of myotubes [17]. Moreover, other soluble factors could participate to EMGstim-induced SCs differentiation [11]. It is worth mentioning that, in FDB myofibers, extracellular ATP increases the expression of the proinflammatory cytokine IL-6 [4,44], which is known to promote muscle cell differentiation [45].

In conclusion, considering the higher efficacy of the EMGstim protocol both in inducing contractile activity in myofibers and differentiation of the myogenic precursors, our data strongly encourage the testing of innovative “noisy” stimulation protocols *in vivo* to better counteract muscle atrophy and favor muscle regeneration during rehabilitation training.

Ethical standards

The experiments comply with the current Italian laws (D.L. 4/3/2014 n.26)

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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