EXPERIMENTAL CELL RESEARCH 323 (2014) 297–313

Available online at www.sciencedirect.com

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Research Article

Defining the role of mesenchymal stromal cells on the regulation of matrix metalloproteinases in skeletal muscle cells

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ARTICLE INFORMATION

Article Chronology:
Received 3 November 2013
Received in revised form
1 March 2014
Accepted 3 March 2014
Available online 12 March 2014

Keywords:
Matrix metalloproteinases (MMPs)
Mesenchymal stromal cells (MSCs)
Myoblast differentiation
Satellite cells
Skeletal fibroblasts

ABSTRACT

Recent studies indicate that mesenchymal stromal cell (MSC) transplantation improves healing of injured and diseased skeletal muscle, although the mechanisms of benefit are poorly understood. In the present study, we investigated whether MSCs and/or their trophic factors were able to regulate matrix metalloproteinase (MMP) expression and activity in different cells of the muscle tissue. MSCs in co-culture with C2C12 cells or their conditioned medium (MSC-CM) up-regulated MMP-2 and MMP-9 expression and function in the myoblastic cells; these effects were concomitant with the down-regulation of the tissue inhibitor of metalloproteinases (TIMP)-1 and -2 and with increased cell motility. In the single muscle fiber experiments, MSC-CM administration increased MMP-2/9 expression in Pax-7^+ satellite cells and stimulated their mobilization, differentiation and fusion. The anti-fibrotic properties of MSC-CM involved also the regulation of MMPs by skeletal fibroblasts and the inhibition of their differentiation into myofibroblasts. The treatment with SB-3CT, a potent MMP inhibitor, prevented in these cells, the decrease of α-smooth actin and type-I collagen expression induced by MSC-CM, suggesting that MSC-CM could attenuate the fibrogenic response through mechanisms mediated by MMPs. Our results indicate that growth factors and cytokines released by these cells may modulate the fibrotic response and improve the endogenous mechanisms of muscle repair/regeneration.

Abbreviations: α-sma, α-smooth muscle actin; bFGF, basal fibroblast growth factor; DIC, differential interference contrast; DM, differentiation medium; ECM, extracellular matrix; EDL, extensor digitorum longus; EDTA, ethylenediaminetetraacetic acid; GFP, green fluorescent protein; HGF, hepatocyte growth factor; IL-1, interleukin -1; MMPs, matrix metalloproteinases; MSC-CM, mesenchymal stromal cell-conditioned medium; MSCs, mesenchymal stromal cells; Pax-7, paired box protein-7; PBS, phosphate buffered saline; PFA, paraformaldehyde; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; ROI, regions of interest; RT, room temperature; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-β; TIMP-1, tissue inhibitor of metalloproteinase-1; TIMP-2, tissue inhibitor of metalloproteinase-2; VEGF, vascular endothelial growth factor

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http://dx.doi.org/10.1016/j.yexcr.2014.03.003
0014-4827/

Introduction

Skeletal muscle has a remarkable ability to regenerate after traumatic injury or disease. This is due to the presence of peculiar stem cells, satellite cells, which lie quiescent under the basal lamina of the muscle fibre until activated in response to injury, when they leave their niche and proliferate before differentiating into myoblasts and fuse into new myofibres [1]. These cells are characterized by the expression of paired box protein (Pax)-7 and myogenic differentiation markers (including Myf5, MyoD, and myogenin) in many muscles [2]. However, the propensity of these cells to repair skeletal muscle is limited in case of extended disease (muscular dystrophies and diabetes), exercise-induced injury [3] or altered use (immobilization, denervation, aging) [4–7]; in these cases, the amount of extracellular matrix (ECM) may increase dramatically relative to muscle fibres, resulting in scarring of the tissue. Muscle fibrosis continues to represent a challenge for clinicians and researchers since it impairs the complete muscle recovery, restricts range of motion, and predisposes to re-injury. Along this line, much attention has been given in the recent years to factors and therapeutic strategies that can improve skeletal muscle healing and regeneration, while reducing scar tissue formation. Studies conducted by our group and others have shown that muscle regeneration can be aided by the administration of growth factors [8–12] and bioactive lipids, including sphingosine 1-phosphate [13–15]. However, these factors are short-lived and more effective methods are required. Emerging evidence suggests that muscle repair can also benefit from stem cell therapy. To this aim, a variety of cell populations have been used, among which bone-marrow-derived mesenchymal stromal cells (MSCs) appear to be attractive candidates for myo-regenerative purposes [16–22]. This because these cells posses some interesting peculiarities for cell therapy, including the relatively easy isolation and expansion in culture, stable phenotype and limited rejection [23]. It is becoming increasingly clear that the MSCs offer benefits beyond their cell replacement potential by providing growth factors and cytokines with multiple effects in the host tissue microenvironment, including neoangiogenesis, the modulation of the endogenous repair mechanisms and prevention of injured cells from the stress response and apoptosis [24–27]. In this context, we have previously demonstrated that MSCs stimulate neonatal cardiomyocyte and skeletal myoblast proliferation [20,28]; these effects are mainly mediated by the secretion of a variety of growth factors and cytokines, including vascular endothelial growth factor (VEGF) [20]. We have also shown that MSC transplantation contributes to skin regeneration by recruiting the local epithelial progenitors to participate in the repair process in the wound [29]. Of interest, the contribution to muscle repair by MSCs and their trophic factors may also involve modulation of fibrosis. Most studies on this field have focused on the role played by MSCs and other stem cells in ameliorating ventricular compliance and improving the cardiac performance after myocardial infarction [19,26,30–35]. However, the role played by these cells in reducing fibrosis in diseased or injured skeletal muscle is less known.

With this in mind, the present study was undertaken to further understand and expand the paracrine activity of MSCs on skeletal muscle repair/regeneration, focusing on the effects of MSCs and their conditioned medium on the expression and activity of matrix metalloproteinases (MMPs), by skeletal C2C12 myoblasts, satellite cells and skeletal fibroblasts derived from mouse skeletal muscle tissue. MMPs are a family of enzymes that selectively digest individual components of ECM; their function is tightly regulated through the action of specific tissue inhibitors of metalloproteinases (TIMPs) and is required for muscle healing, by reducing fibrosis and promoting myogenic cell migration through the ECM to the site of injury [36–41]. We found that factors released by MSCs exert potent anti-fibrotic effects via their ability to regulate the balance of MMP-2 and MMP-9/TIMP-1 and-2 production by the assayed muscle cells and improve satellite cell migration and differentiation, thus providing new insights into the potential role of MSC-cell therapy in muscle regenerative medicine.

Material and methods

Ethics statement

All animals' manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986; 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the University of Florence. The ethical policy of the University of Florence conforms to the Guide for the care and use of laboratory animals of the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance No. A5278-01). The protocols were communicated to local authorities and to Italian Ministry of the Health; according to the Italian law (Art.7/D.lgs 116/92); such procedure doesn't require Ministry authorization. The animals were housed with free access to food and water and maintained on a 12 h light/dark cycle at 22 °C room temperature (RT). All efforts were made to minimize the animal suffering and the number of animals sacrificed. Animals were killed by decapitation.

Cell culture and treatments

Mouse bone marrow mesenchymal stromal cells (m-MSCs) were isolated from femura and tibiae of male C2F1 mice, following the Dobson's procedure [42], expanded *in vitro* and characterized as reported previously [20]. In some experiments, these cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2, in C2C12 myoblast differentiation medium (myoblast DM) or in muscle satellite culture medium or in NIH3T3 cell or primary skeletal fibroblast culture medium for 24 h and the culture medium (MSC-derived conditioned medium, MSC-CM) was harvested and used for culturing C2C12 myoblasts, satellite cells, single muscle fibres, NIH3T3 or primary skeletal fibroblasts to assess MSC paracrine effects.

Transgenic bone marrow green fluorescent protein (GFP)-labeled MSCs (GFP-MSCs) were isolated from male GFP transgenic Lewis rats (RRRC, Missouri, USA), expanded and characterized as described previously [43]. GFP- MSCs were analyzed for green fluorescence intensity at different passages in culture as well as for the expression of particular cell surface molecules using flow cytometry procedures: CD45-CyChromeTM, CD11b-FITC (in order to quantify hemopoietic-monocytic contamination), CD90-PE, CD73-PE, CD44-PE (BD Pharmingen, San Diego, CA, USA).

Murine C2C12 skeletal myoblasts obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Sigma, Milan, Italy) at 37 °C in a humidified atmosphere of 5% CO_2 till reaching 80% confluence. Then, they were shifted in differentiation medium (myoblast DM), containing 2% horse serum (HS, Sigma) and cultured for 24 h (control). In some experiments the cells were co-cultured with m-MSCs or rat GFP-MSCs at a 2:1 ratio for 24 h in myoblast DM. In parallel, C2C12 myoblasts were cultured in a bottom chamber of a polycarbonate transwell system (Millipore, Billerica, MA, USA) with MSCs put in top chamber, or exposed to MSC-CM (conditioned medium by MSCs cultured in myoblast DM for 24 h) in order to assess the paracrine effects of MSCs.

Single muscle fibres and satellite cells were isolated from *Extensor Digitorum Longus* (EDL) muscles carefully removed from anesthetized (with 50 μg/g zolazepam Tiletamine) young adult male Swiss mice (25–30 g) essentially as described previously [14]. Briefly, EDL muscles, soon after isolation, were digested in 0.2% collagenase type-I in DMEM (Sigma) and then transferred to a Petri dish containing serum-free DMEM, in which single muscle fibres were isolated from the muscles, by means a gentle mechanical trituration with a Pasteur pipette. The intact, viable muscle fibres were collected and subsequently cultured individually in Matrigel (BD Biosciences, San Jose, CA, USA) treated 24-well plates, at 37 °C in a humidified atmosphere of 5% CO_2, in satellite cell proliferation medium containing DMEM, 20% FBS, 10% HS, 0.5% chicken embryo extract (Sera Laboratories International Ltd, Horsted Keynes, UK) plus 1% penicillin/streptomycin (Sigma) for 48 h. In some experiments after 24 h of culture, the myofibres were shifted in MSC-CM (conditioned medium by MSCs cultured in satellite proliferation medium for 24 h) for further 24 h. In other experiments, after 48 h of culture, the myofibres were removed and the derived satellite cells were expanded in culture in proliferation medium (control). In some experiments the cells (P1) were treated with MSC-CM (conditioned medium by MSCs cultured in satellite culture medium for 24 h) for 24 h and 48 h (at this time point, the MSC-CM was changed after 24 h). Satellite cells were assayed for Pax-7 expression by confocal immunofluorescence analysis to testify high purity of the culture.

Primary murine skeletal fibroblasts were prepared from EDL skeletal muscles, after the single myofibres were isolated and cultured in satellite cell proliferation medium as described above. In particular the cells sprouting from the 48 h cultured single myofibres, were detached after reaching 70% of confluence, with 0.05% trypsin–0.03% ethylenediaminetetraacetic acid (EDTA; Sigma) for 5 min at 37 °C, washed with phosphate buffered saline (PBS), and then seeded in a culture plate with fresh medium. After 20 min the non-adherent cells were collected and plated for satellite cell culture preparation, while the adherent ones (P0) were washed and shifted in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin (Sigma) to be expanded at 37 °C in a humidified atmosphere of 5% CO_2. Aliquots of cells at P1 culture passage were seeded on glass coverslips and assayed for vimentin and von Willebrand immunophenotype by confocal microscopy to confirm their stromal nature and to test the degree of purity of the cell cultures, which usually was 92–98%. To promote fibroblast-myofibroblast transition the cells were cultured in low serum (2% FBS) culture medium. In some experiments the fibroblastic cells (P1) were co-cultured with rat GFP-MSCs at a 1:1 ratio for 24 h in fibroblast low serum culture medium or were exposed to MSC-CM (conditioned medium by MSCs cultured in skeletal fibroblast low serum culture medium for 24 h) for 24 h.

Murine NIH3T3 fibroblasts obtained from ATCC were routinely cultured in DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2. To promote fibroblast-myofibroblast transition the cells were cultured in low serum (2% FBS) culture medium. In some experiments the cells were co-cultured with rat GFP-MSCs at a 1:1 ratio or treated with MSC-CM (conditioned medium by MSCs cultured in fibroblast low serum culture medium for 24 h) for 24 h in order to assess the paracrine effects of MSCs.

In parallel experiments C2C12 cells and primary murine skeletal fibroblasts were cultured in the presence of 5 and 10 μM of a potent inhibitor of MMP-2 and MMP-9, SB-3CT (Sigma), as previously reported [44].

Confocal immunofluorescence

The different cell types grown on glass coverslips were fixed with 0.5% buffered paraformaldehyde (PFA) for 10 min at RT. After permeabilization with cold acetone for 3 min, the fixed cells were blocked with 0.5% bovine serum albumin (BSA; Sigma) and 3% glycerol in PBS for 20 min and then incubated with the following primary antibodies: rabbit polyclonal anti-Ki67 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit polyclonal anti-MMP-2 (1:200; Abcam, Cambridge, UK); rabbit polyclonal anti-MMP-9 (1:100; Abcam); rabbit polyclonal anti-TIMP-1 (1:50; Bioss Inc, Woburn, MA, USA); rabbit polyclonal anti-TIMP-2 (1:20; Abcam); mouse monoclonal anti-Pax-7 (1:100; Santa Cruz Biotechnology); rabbit polyclonal anti-MyoD (1:100; Santa Cruz Biotechnology); rat monoclonal anti-laminin 2 α (1:200; Abcam); goat polyclonal anti-vimentin (1:40; Sigma); rabbit polyclonal anti- von Willebrand factor (1:200; Sigma); mouse monoclonal anti- α smooth muscle actin (α-sma, 1:100; Abcam) and rabbit polyclonal anti-type-I collagen (1:50; Santa Cruz Biotechnology) overnight at 4 °C. The immunoreactions were revealed by incubation with specific anti-rabbit, anti-mouse, anti-rat or anti-goat Alexa Fluor 488- or 568-conjugated IgG (1:200; Molecular Probes, Eugene, OR, USA) for 1 h at RT. In some experiments, counterstaining was performed with propidium iodide (PI, 1:30; Molecular Probes) for 30 s to reveal nuclei. Negative controls were carried out by replacing the primary antibodies with non-immune serum; cross-reactivity of the secondary antibodies was tested in control experiments in which primary antibodies were omitted. After washing, the coverslips containing the immunolabeled cells were mounted with an antifade mounting medium (Biomeda Gel mount, Electron Microscopy Sciences, Foster City, CA, USA) and observed under a confocal Leica TCS SP5 microscope (Leica Microsystems, Mannheim, Germany) equipped with a HeNe/Ar laser source for fluorescence measurements and with differential interference contrast (DIC) optics. Observations were performed using a Leica Plan Apo 63 × /1.43NA oil immersion objective. Series of optical sections (1024 × 1024 pixels each; pixel size 204.3 nm) 0.4 μm in thickness were taken through the depth of the cells at intervals of 0.4 μm. Images were then projected onto a single 'extended focus' image. When needed, a single optical fluorescent section and DIC images were merged to view the precise distribution of the immunostaining. Densitometric analyses of the intensity of MMP-2, MMP-9, TIMP-1, TIMP-2, MyoD, type-I collagen and α-sma fluorescent signals were

performed on digitized images using ImageJ software (http://rsbweb.nih.gov/ij) in 20 regions of interest (ROI) of 100 μm^2 for each confocal stack (at least 10). The number of C2C12 cells with Ki67 positive nuclei was evaluated in 10 random 200 × 200 μm square microscopic fields (60× ocular) in each cell preparation and expressed as percentage of the total cell number. The number of Pax-7 positive satellite cells sprouting from single muscle fibres (at least 10) was evaluated on 10 random 200 × 200 μm^2 optical square fields (60×) under the confocal Leica TCS SP5 microscope.

Flow cytometry

Flow cytometry analysis was performed to immune-phenotypically characterize C2C12 cell and mouse MSC populations with the aim to distinguish the two cell populations, when co-cultured, on the basis of their cell surface antigen, essentially as described previously [20]. Briefly, mouse MSCs and C2C12 cells recovered from flask by trypsin-EDTA treatment, were re-suspended in flow cytometry buffer consisting of CellWASH (0.1% sodium azide in PBS; Becton Dickinson, San Jose, CA, USA) with 2% FBS and incubated with PE- conjugated monoclonal antibodies (BD Pharmingen) against the myoblastic cell marker CD34 [45]; 7-aminoactinomycin AAD (7-AAD; BD Pharmingen) was added in order to exclude dead cells from the analysis. Flow cytometric acquisition was performed by collecting 10^4 events on a FACSCanto (Becton Dickinson) instrument and data were analyzed on DIVA software (Becton Dickinson).

Immunomagnetic cell separation

C2C12 myoblasts and mouse MSCs were separated after 24 h co-culture using MACS micro beads technology (Miltenyi Biotec, Bologna, Italy) essentially as reported previously [20]. In particular, the co-cultured cells were recovered by trypsin-EDTA treatment resuspended in Buffer containing 0.5% BSA and 2 mM EDTA and incubated with CD34 PE-conjugated antibody (BD Pharmingen) following manufacturer's instructions. Cells were then incubated with anti-PE MicroBeads and separated on MS MACS column following manufacturer's instructions (Miltenyi Biotec). The CD34 positive (C2C12 cells) cell fraction was then re-analyzed by flow cytometry to assess cell viability and purity and processed for Western blotting analysis. C2C12 cells in single culture were subjected to the same treatments of those in co-culture and used as control ($CD34^+$ C2C12 single culture).

Western blotting

Cells were resuspended in appropriate volume of cold Cell Extraction Buffer (10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulphate (SDS), 0.5% deoxycholate; Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with 50 μl/ml Protease Inhibitor Cocktail (Sigma) and 1 mM Phenylmethanesulfonyl fluoride, PMSF (Sigma). Upon centrifugation at 13.000*g* for 10 min at 4 °C, the supernatants were collected and the total protein content was quantified by Bio-Rad protein assay (Bio-Rad Laboratories S.r.l., Milan, Italy) following the manufacturer's instructions. Forty micrograms of total proteins were electrophoresed on NuPAGE® 4–12% Bis-Tris Gel (Invitrogen; 200 V, 40 min) and blotted onto polyvinylidene difluoride (PVDF) membranes (Invitrogen; 30 V, 1 h). The membranes were blocked with Blocking Solution included in the Western Breeze®Chromogenic Western Blot Immunodetection Kit (Invitrogen) for 30 min at RT on rotary shaker and incubated overnight at 4 °C with rabbit polyclonal anti-MMP-2 (1:2000; Abcam), rabbit polyclonal anti-MMP-9 (1:1000; Abcam), mouse monoclonal anti-α-sma (1:1000; Abcam) and rabbit polyclonal anti α-tubulin (1:1000; Millipore) antibodies, assuming α-tubulin as control invariant protein. Immunodetection was performed as described in the Western Breeze®Chromogenic Immunodetection protocol (Invitrogen). Densitometric analysis of the bands was performed using ImageJ software (http://rsbweb.nih.gov/ij) and the values normalized to α-tubulin.

Gelatinase assay

The MMP activity in myoblasts and fibroblastic cells was evaluated using EnzChek® Gelatinase/Collagenase Assay Kit (Molecular Probes) which provides a highly quenched, fluorescein-labeled gelatin (DQ^{TM} gelatin). Upon proteolytic digestion, the green fluorescence of the gelatin, is revealed and can be used to measure enzymatic activity. In particular, the wells of a 96-well microplate reader were coated with 25 μg/ml of DQ^{TM} gelatin following the manufacturer's instructions; C2C12 or fibroblastic cells in different experimental culture conditions or the sole MSC-CM were added to the coated wells and incubated at 37 °C in a humidified atmosphere of 5% CO_2, for 24 h before reading the fluorescent intensity by using a multi-well scanning spectrophotometer (ELISA reader; Amersham, Pharmacia Biotech, Cambridge, UK) at a wavelength of 515 nm. In parallel experiments, C2C12 cells were seeded onto glass coverslips previously coated with fluorescein-conjugated DQ^{TM} gelatin (25 μg/ml) cultured for 24 h in DM or MSC-CM and then observed under a confocal Leica TCS SP5 microscope (Leica Microsystems).

In vitro cell migration assay

Cell migration assay was performed according to methods previously described [46], with minor modifications. Briefly, C2C12 cells (3×10^5) were cultured on glass coverslips coated with type-I collagen (Sigma) until 70% confluent in a complete medium. Cells were then shifted in DM or in CM-MSC in the presence or absence of SB-3CT (10 μM, Sigma), and cultured for 24 h before an artificial wound was created in the monolayer using a sterile plastic pipette tip. A perpendicular mark was placed across each scratch on the external surface of the glass coverslips to allow quantitative analysis. Repopulation of the wounded area was observed under phase contrast microscopy at 0 and 24 h after scraping. The average distance migrated by the cells was evaluated as the difference of the cell front relative to the 0 h time point, using ImageJ software (http://rsbweb.nih.gov/ij). After the analysis of cell migrating, the cells were fixed in 0.5% PFA, stained with Alexa Fluor 488 labelled-phalloidin (1:40 for 20 min at RT; Molecular Probes) to reveal actin filaments and observed by confocal Leica microscopy.

Time-lapse videomicroscopy

Single muscle fibres and the derived satellite cells were analyzed for 24 h by time-lapse videomicroscopy (1 frame/5 min, exposure time 0.5 s) using an inverted phase-contrast (Nikon, Tokyo, Japan) equipped with a 10× objective and a cooled video camera

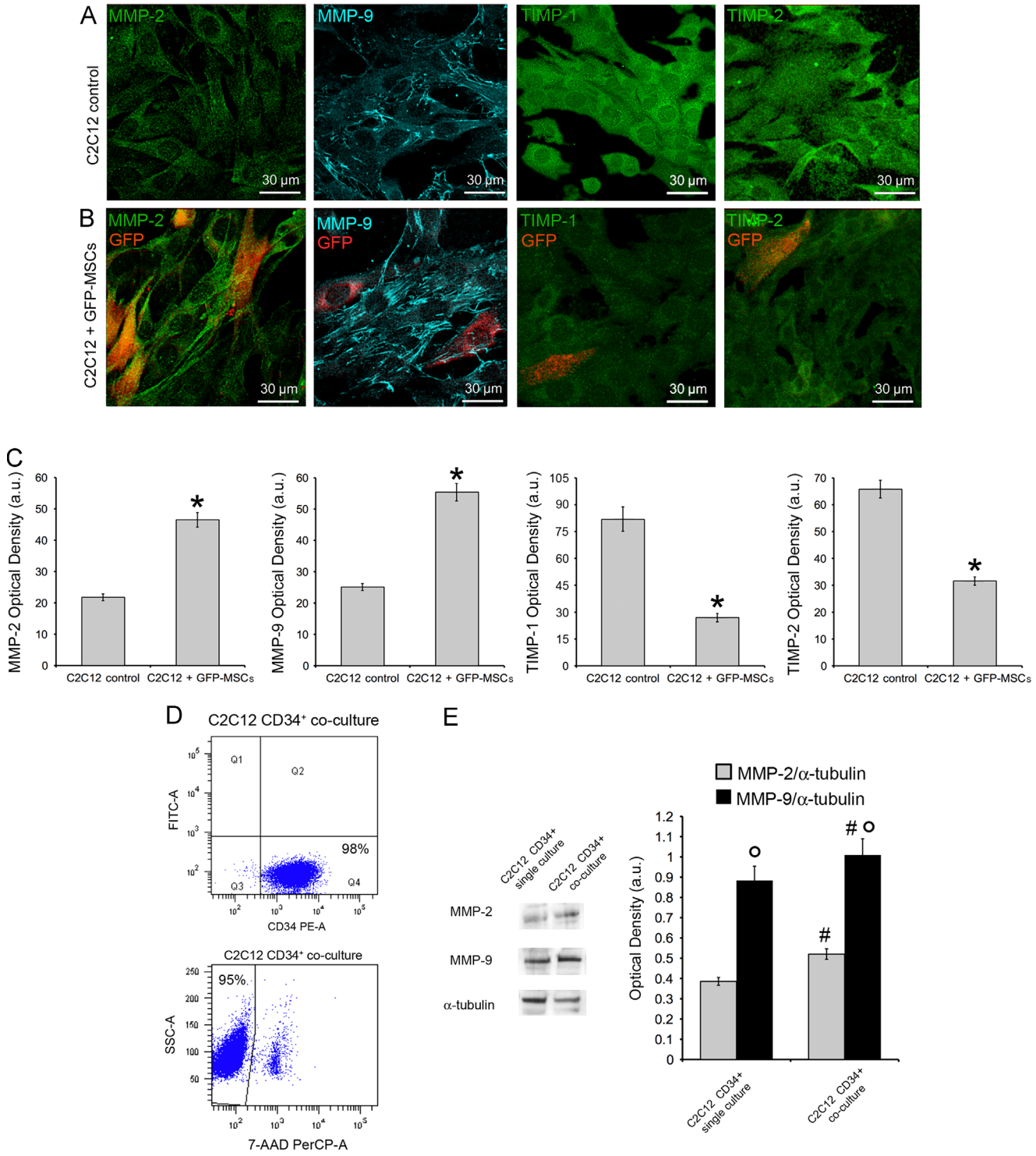


Fig. 1 – Expression of MMP-2 and MMP-9 and their inhibitors (TIMP-1 and TIMP-2) in C2C12 myoblasts in single and co-culture with MSCs. (A), (B) Representative immunofluorescence confocal images of C2C12 cells in (A) single (control) and (B) co-culture with GFP-MSCs (red pseudocolor) for 24 h, immunostained with antibodies against MMP-2 (green), MMP-9 (cyan), TIMP-1 (green) and TIMP-2 (green). (C) Densitometric analyses of the intensity of the specific fluorescence signals performed on digitized images. (D) Flow cytometric analysis of CD34 antigen expression in C2C12 myoblasts in co-culture, after immunomagnetical separation from m-MSCs, using anti-CD34 antibody. Purity of C2C12 cell fraction after separation (C2C12 CD34$^+$ co-culture) was 98%. Viability, measured as 7-AAD negative cells, was 95%. (E) Western blotting analysis of MMP-2 and MMP-9 expression on CD34$^+$ C2C12 cells in single (C2C12 CD34$^+$ single culture) and co-culture (C2C12 CD34$^+$ co-culture). The densitometric analysis of the bands normalized to α-tubulin is reported in the histogram. Data are representative of at least three independent experiments with similar results. Significance of differences in (C) and (E) was evaluated by Student's *t* test: * $p<0.05$ *vs* C2C12 control, $^{\#}p<0.05$ *vs* C2C12 CD34$^+$ single culture; $^{\circ}p<0.05$ *vs* MMP-2.

equipped with a motorized filter wheel and its dedicated digital recording software (Chroma CX3, DTA, Cascina, Italy).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.yexcr.2014.03.003.

Myotubes formation

Myotubes were also observed and their number evaluated after 48 h satellite cell culture in proliferation medium (control) or in MSC-CM, in 10 random 691.200 μm^2 optical square fields (20× ocular) under an inverted phase contrast microscope Nikon Diaphot 300 (Nikon) in each cell preparation (at least 10).

Statistical analysis

Data were reported as mean ± s.e.m. Statistical significance was determined by one-way ANOVA and Newman–Keuls multiple comparison test or Student's *t* test (comparison between two populations). A p value ≤ 0.05 was considered significant. Calculations were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA).

Results

MSCs up-regulate MMP expression and activity in cultured myoblastic cells

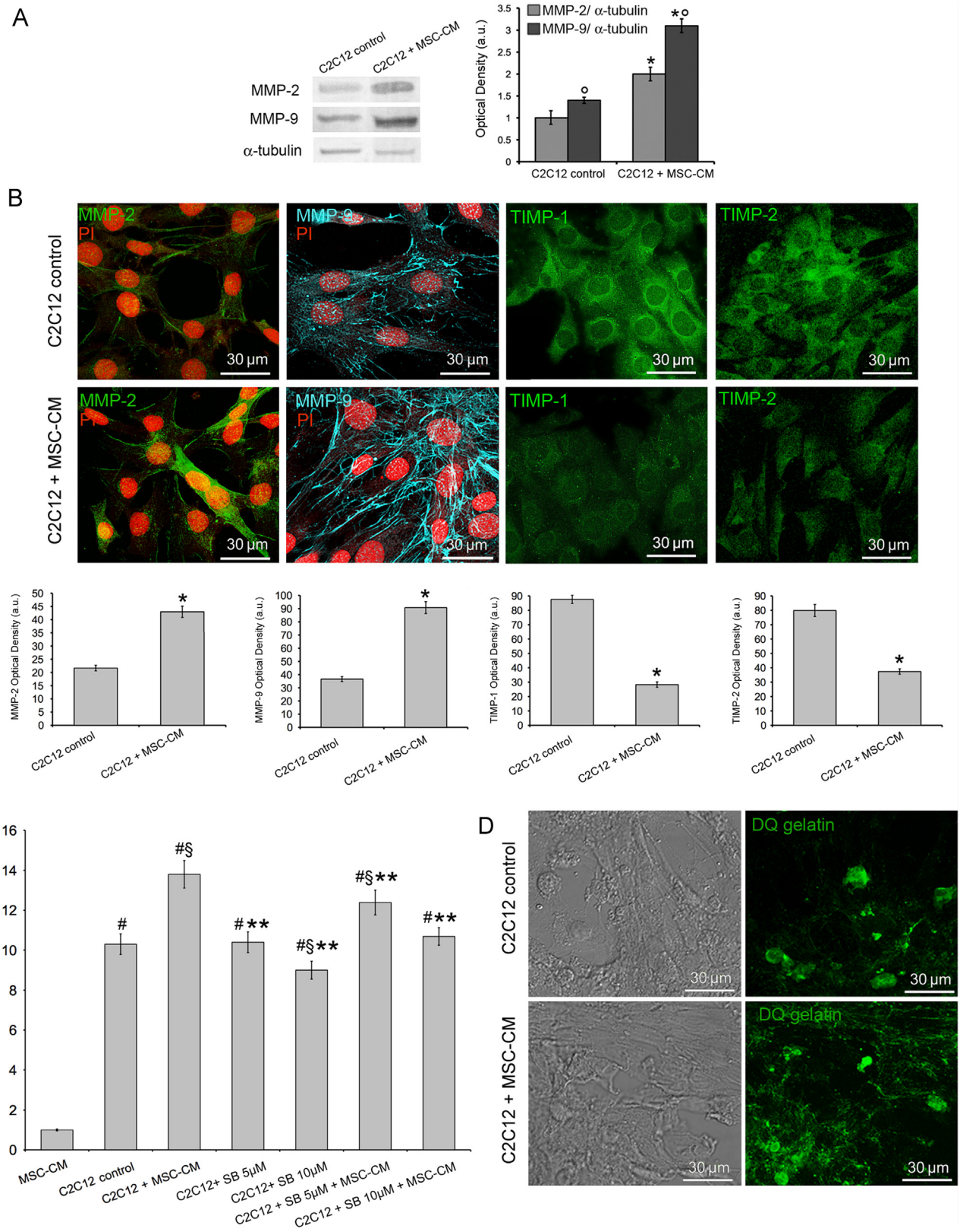
To investigate the interactions between MSCs and skeletal myoblasts, we first co-cultured C2C12 cells with MSCs isolated from bone marrow of green fluorescent protein (GFP) transgenic Lewis rats (GFP-MSCs) or C2F1 mice (m-MSCs) for 24 h. As shown in Fig. 1, C2C12 cells cultured alone expressed relatively high levels of MMP-9 and much lower levels of MMP-2, in agreement with our previous observations [31]. The co-presence of GFP-MSCs or m-MSCs in the culture, beside stimulating myoblast proliferation (81.2 ± 6% and 61.1 ± 5% of the myoblast nuclei resulted Ki67 positive in the co-culture and single culture, respectively; Student's Test, $p<0.05$) enhanced MMP-9 and MMP-2 production and down-regulated the expression of their inhibitors TIMP-1 and -2, as judged by confocal immunofluorescence (Fig. 1A–C) and Western blotting analysis performed in C2C12 cells immunomagnetically separated from m-MSCs using antibodies against specific myoblastic cell markers (CD34) (Fig. 1D and E). In particular, both MMP-2 and MMP-9 had similar subcellular distribution in the myoblastic cells and appeared linearly organized along the cytoskeletal filaments (Fig. 1A and B). Their inhibitors, TIMP-1 and TIMP-2, appeared evenly distributed within the cytoplasm (Fig. 1A and B). To monitor MSC-mediated paracrine effects, C2C12 cells were co-cultured with either GFP-MSCs or m-MSCs using a transwell system or treated with MSC-conditioned medium (MSC-CM) for 24 h. It was found that the expression levels of both MMP-2 and MMP-9 were increased by approximately two-folds after C2C12 cells were exposed to factors secreted by MSCs (Fig. 2A and B). In contrast, the expression of TIMP-1 and -2 were reduced (approximately by three and two-folds, respectively) in C2C12 cells treated with MSC-CM (Fig. 2B). With DQ™ gelatin degradation assay, we also demonstrated that gelatinase (MMP-2 and MMP-9) activities of C2C12 cells were increased after exposure to MSC-CM (Fig. 2C and D), indicating that these cells synthesized functional MMPs. These data correlated with the increased cell motility observed in MSC-CM-treated cells as compared with control cells. In fact, utilizing a scrape migration assay, we found that the addition of MSC-CM significantly increased myoblast migration distance (Fig. 3A–D,G). Interestingly, the incubation of the cells with SB-3CT, a potent MMP-2/9 inhibitor (Fig. 2C), inhibited the effects of the MSC-CM on cell motility, reducing the migration distance into the artificial wound (Fig. 3A–D,G). It is well known that a finely tuned balance between actin filament polymerization/depolymerization is required for cell migration [47]. Accordingly, MSC-CM treated cells, compared to the untreated controls, showed a more elongated shape along with a better organized cytoskeleton with robust stress fibers and expressed raft-like structures (*i.e.* microspikes and lamellipodia) at the migratory edge (Fig. 3E and F), suggestive of increased cell migration. By contrast, the cells incubated with SB-3CT (10 μM), revealed a round-shaped morphology and reduced cytoskeletal assembly (Fig. 3E and F).

Altogether these data suggested that MSCs were able to regulate MMP expression and function in skeletal myoblasts through the release of paracrine factors.

Fig. 2 – MMP-2 and MMP-9 expression/activity and TIMP-1 and TIMP-2 expression in C2C12 myoblasts exposed to factors secreted by MSCs. C2C12 myoblasts were cultured alone (C2C12 control) or exposed to MSC-conditioned medium (C2C12+MSC-CM) for 24 h in the presence or absence of a potent MMP2/9 inhibitor, SB-3CT (SB, 5 and 10 μM). (A) Western blotting analysis of MMP-2 and MMP-9 expression. The densitometric analysis of the bands normalized to α-tubulin is reported in the histogram. (B) Representative immunofluorescence confocal images of C2C12 cells in the indicated experimental conditions, immunostained with antibodies against MMP-2 (green), MMP-9 (cyan), TIMP-1 (green) and TIMP-2 (green). In some images, nuclei are counterstained in red with propidium iodide (PI). Densitometric analyses of the intensity of the fluorescence signals of each specific marker performed on digitized images, are reported in the histograms. (C), (D) Gelatinase assay. C2C12 myoblasts were seeded on a fluorescein-labeled gelatin substrate (DQ™ gelatin) and cultured in the indicated experimental conditions. (C) Spectrophotometrical quantification of the DQ™ gelatin fluorescence intensity revealed after proteolytic digestion of the gelatin by MMP gelatinases. MSC-CM refers to the sole MSC-CM added to the DQ™ gelatin substrate. (D) C2C12 cells seeded onto DQ™ gelatin coated glass coverslips, cultured as indicated and observed under a confocal fluorescent microscopy. The micrographs in grey scale are representative DIC images of C2C12 cells in the indicated experimental conditions. In green, the gelatin fluorescence intensity. Data are representative of at least three independent experiments with similar results. Significance of differences in (A) and (B) was evaluated by Student's *t* test: *$p<0.05$ *vs* C2C12 control; °$p<0.05$ *vs* MMP-2; significance of differences in (C) was evaluated by one-way ANOVA and Newman–Keuls multiple comparison test: #$p<0.05$ *vs* MSC-CM; §$p<0.05$ *vs* C2C12 control; **$p<0.05$ *vs* C2C12+MSC-CM.

MSCs up-regulate MMP expression and promote myogenic differentiation in satellite cells

To further verify and extend the effects of MSCs on MMPs in myoblastic cells, we next evaluated whether MSC-CM influenced the expression of MMP-2 and MMP-9 in satellite cells isolated from single muscle fibres. By time-lapse videomicroscopy it was found that small mononucleated cells moved under the basal lamina of the muscle fibre and reached the surface of the host myofibre where they rapidly proliferated (Fig. 4A and B; Supplemental files



1,2). These cells expressed Pax-7 (Fig. 5), which is considered a cell marker of satellite cells that are highly myogenic and self-renewable [2]. After the exposure to factors contained in the MSC-CM, MMP-2 and MMP-9 were up-regulated in Pax-7^{+} satellite cells (Fig. 5A and B), whereas the levels of TIMP-1 and TIMP-2 were down-regulated (Fig. 5B). In these experimental conditions, these cells also showed

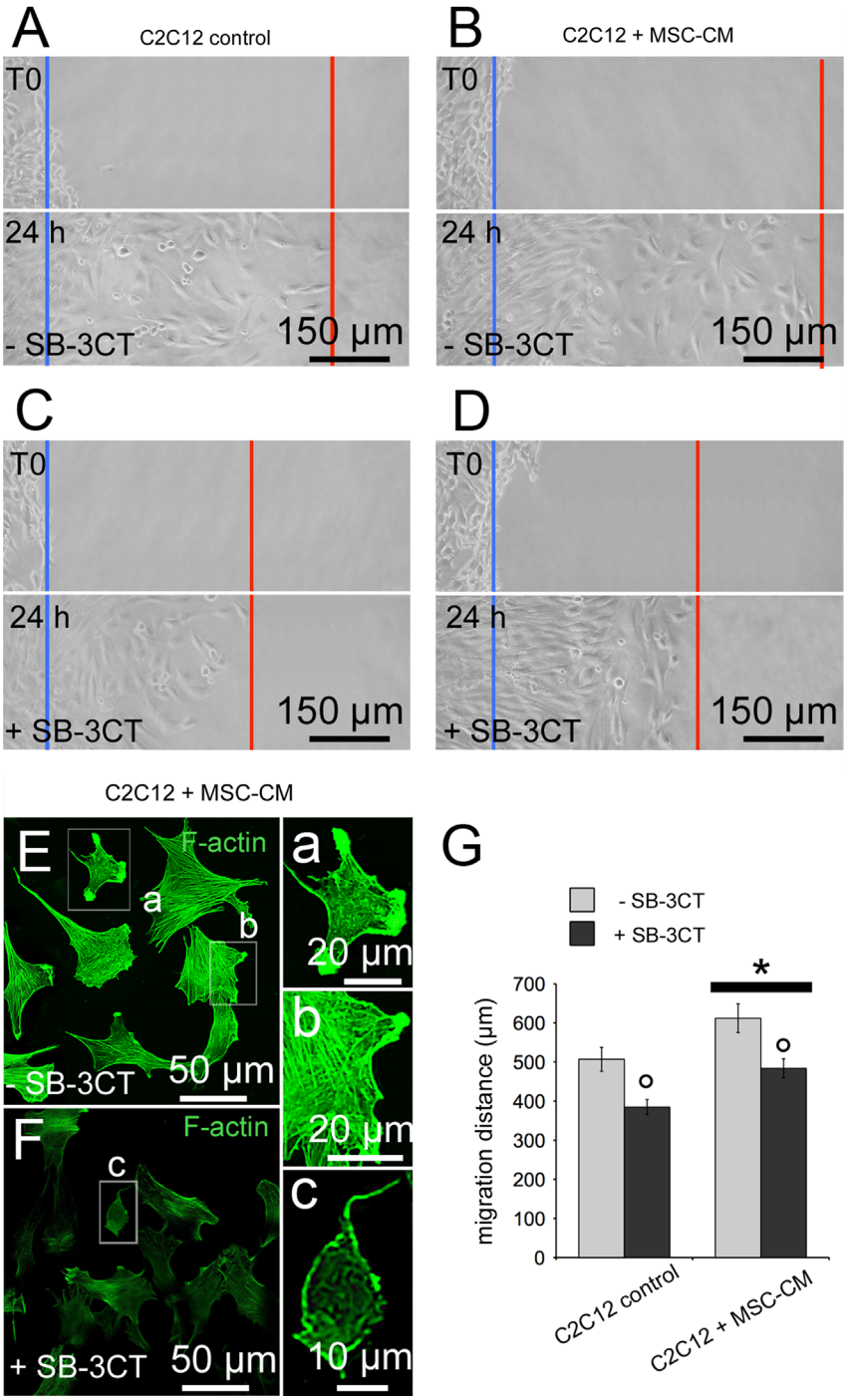


Fig. 3 – Migration assay. (A)–(D) C2C2 cells were cultured on glass coverslips coated with type-I collagen in differentiation medium (DM, control) or treated with MSC-CM (C2C12+MSC-CM) in the presence (+) or absence (−) of a specific MMP2/9 inhibitor, SB-3CT (10 µM), for 24 h after having removed the cells from an half of the coverslip using plastic pipette tip to allow the cells to migrate into the scrape area. The blu line denotes the edge of the scrape at time 0 (T0), and the red line denotes migration front after 24 h. Migration distance was calculated as the difference between red and blue lines in each field of view. (E) and (F) In some experiments, the cells after migration, were fixed and stained with Alexa488-conjugated phalloidin to visualize actin filaments (F-actin). a-c, magnification of ROI indicated in (E) and (F), showing features of migrating [actin microspikes (a,b) and lamellipodia (a)] and non migrating cells (c). (G). Quantitative analysis of cell migration. The values are the media of three independent experiments. Significance of differences was evaluated by Student's *t* test: $^{*}p<0.05$ *vs* C2C12 control; $^{\circ}p<0.05$ *vs* –SB-3CT.

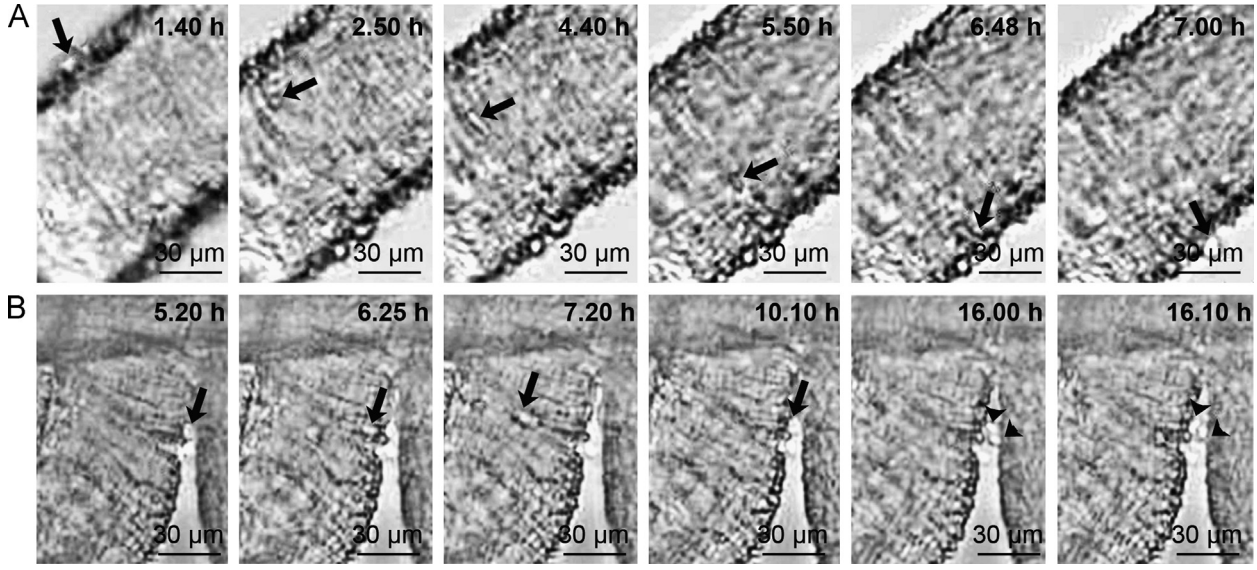


Fig. 4 – Satellite cell activation in skeletal muscle fibres. (A), (B) Time-lapse videomicroscopy analysis of skeletal muscle fibres isolated from EDL muscle cultured in satellite cells growth medium (1 frame/5 min). Arrows indicate migration of a satellite cell under the basal lamina of the muscle fibre, whereas arrowheads (B) point to satellite cell proliferation at the surface of the host myofibre.

increased motility, proliferation and differentiation potentials. This mostly on the basis of the following data showing that after the addition of MSC-CM: (i) a higher number of Pax-7^{+} satellite cells sprouted from the single fibres (Fig. 6A); (ii) there was an increased fragmentation of the basal lamina around myofibres, as judged by laminin 2α immunostaining (Fig. 6A), and; (iii) the sprouting myoblasts exhibited increased levels of MyoD, a marker of satellite cell activation [2] (Fig. 6A) associated with a higher tendency to fuse into multinucleated myotubes (Fig. 6B). These data taken together suggested that satellite cell migration and differentiation potentials could be promoted by MSC-CM, possibly by regulating MMP activity.

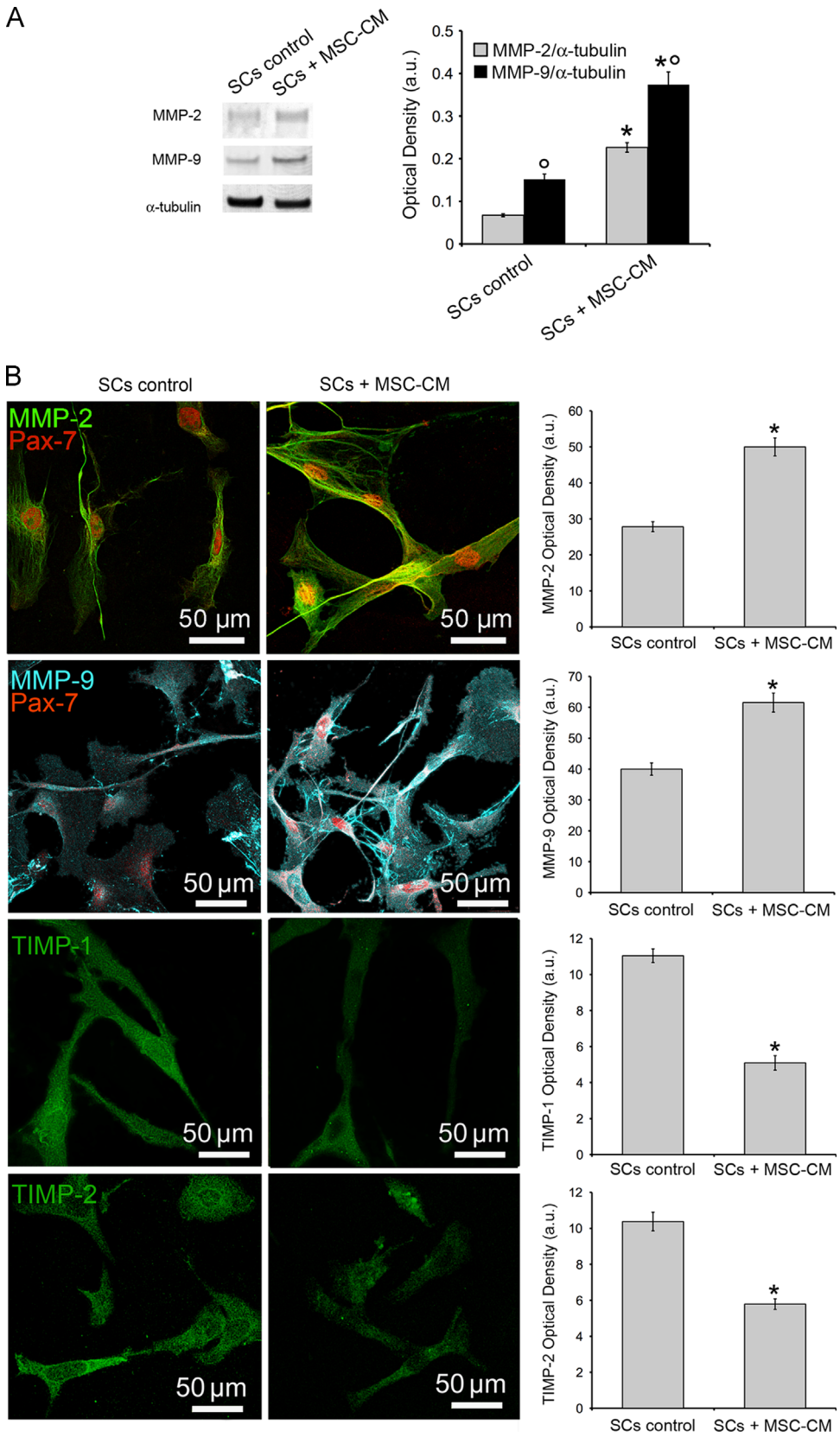
MSCs up-regulate MMP expression in skeletal fibroblasts and inhibit fibroblast-myofibroblast transition

Beside myoblasts, skeletal fibroblasts can also regulate ECM remodelling. Several lines of evidence suggest that this role is accomplished through two major mechanisms: secretion of ECM-degrading enzymes and differentiation into myofibroblasts, producing collagen and others ECM proteins [48]. On this background, we investigated whether skeletal fibroblasts could be a target of MSC-CM. Fibroblasts were isolated from mouse skeletal muscle and characterized at the first passage using morphological (flat and spindle-shaped with several projecting processes) and immunocytochemical (vimentin-positive and von Willebrand factor-negative) criteria (Fig. 7A). In co-culture with GFP-MSCs, and after exposure to MSC-CM, skeletal fibroblasts and NIH3T3 fibroblastic cell line (data not shown) showed enhanced expression and activity of MMP-2 and MMP-9 (Fig. 7B–E), concomitant with reduced levels of TIMP-1 and TIMP-2 (Fig. 7C and D). This effect was associated with a decrease in α-smooth muscle actin (α-sma) (Fig. 7B and C), the most reliable marker of myofibroblasts, and type-I collagen expression in the cells cultured with GFP-MSCs or treated with MSC-CM (Fig. 8A and B), indicating that the anti-fibrotic potential of MSCs on skeletal muscle fibroblasts could be related not only to the stimulation of MMPs functions but also to the inhibition of their ability to undergo fibroblast-myofibroblast transition and accumulate collagen. Of interest, we also demonstrated that the pre-treatment with SB-3CT prevented the increase in MMP activity induced by MSC-CM (Fig. 7E) and inhibited MSC-CM decreased α-sma and type-I collagen expression (Fig. 8A and B), suggesting that the preventive effects of paracrine factors from MSCs on fibroblast differentiation could be, at least in part, associated with MMP-pathway.

Discussion

The ECM provides the structural support of tissues and organs by serving as a scaffold for cells and determines their mechanical properties. Its homeostasis is dependent on a fine coordination between a family of proteolytic enzymes that selectively digest individual components of ECM, MMPs, and their specific tissue inhibitors (TIMPs). Over 25 members of the MMP family and 4 TIMPs family have been identified so far [41]. These molecules are constitutively expressed by a wide range of cell types from connective tissues, inflammatory and stem cells and are regulated by a number of growth factors, cytokines and chemokines. MMP/TIMP balance plays pivotal roles in normal functioning tissues during growth, development and regeneration. Previous studies have, in fact, demonstrated that dismantling the ECM by MMP gelatinases, MMP-2 and MMP-9, is required for satellite cell activation and efficient skeletal muscle regeneration [1,38,49–52]. Moreover, their inhibition negatively impact skeletal muscle healing [52]. MMP expression and function may also be associated with aberrant tissue repair response and disease progression in many tissues, including skeletal muscle [37,53]. In particular, increased MMP-2 and MMP-9 activity has been found in severe muscular dystrophies, inflammatory myopathies, disuse atrophy, aging and injured muscle and is associated with the chronic persistence of inflammatory infiltrate, continuous cycles of myofibre degeneration and regeneration and massive proliferation

of fibroblasts [5,54]. In these conditions, the formation of a permanent fibrotic tissue surrounding the myofibres leads to a significant impairment of the muscle compliance and function and increases the susceptibility to re-injury. Of interest, the introduction of exogenous MMP-1 into the previously formed scar tissue of injured mouse gastrocnemius muscle results in a significant reduction of collagen content and improvement in the number of regenerating myofibres [55,56]. These data suggest



that increased MMP activity into the fibrotic tissue may be able to rescue the pathological muscle, making the tissue microenvironment more hospitable and conductive to cell regeneration. The mechanisms behind these effects are related to the fact that these enzymes disrupt ECM and the components of myofibre basement membrane that hinder myoblasts to reach the site of injury and differentiate and fuse into multinucleated fibers during muscle repair/regeneration. Along this line, we have shown in the present

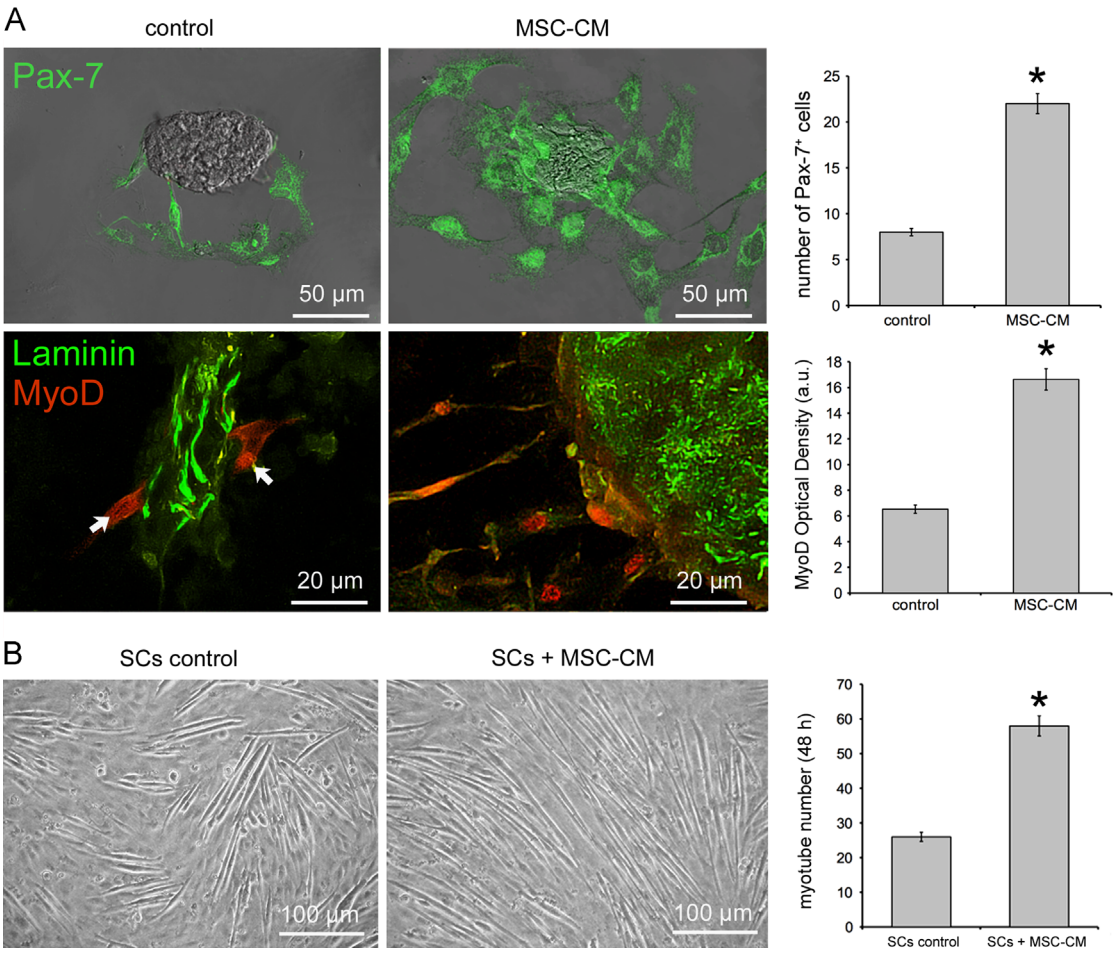
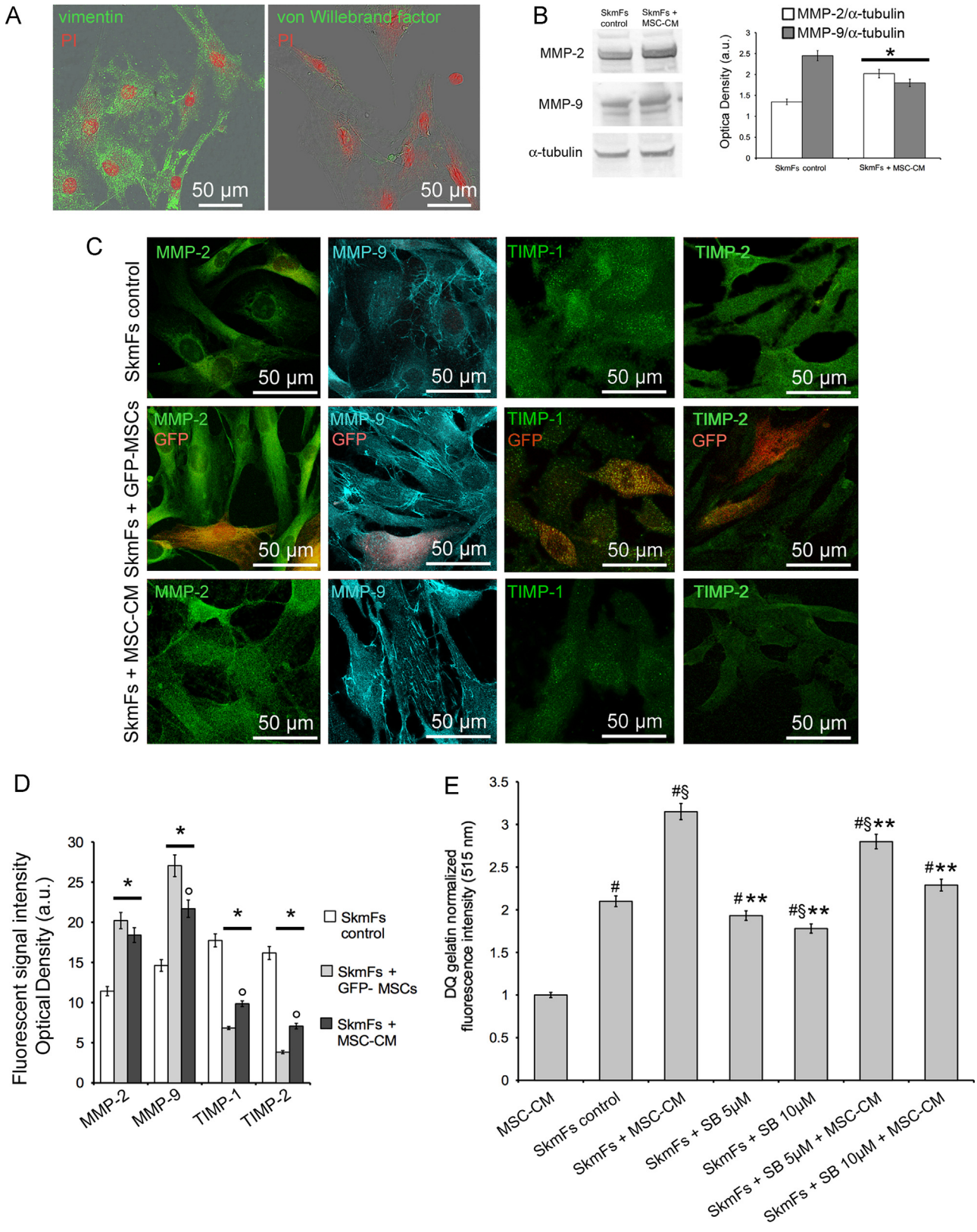


Fig. 6 – Activation and differentiation of satellite cells from single skeletal muscle fibres exposed to factors secreted by MSCs. (A) *Upper panels*: representative superimposed DIC and confocal fluorescence images of satellite cells (SCs) sprouting from a single skeletal muscle fibre cultured in satellite proliferation medium (control) or exposed to MSC-conditioned medium (MSC-CM) for 24 h, fixed and immunostained with antibodies against Pax-7 (green). *Lower panels*: representative confocal fluorescence images of SCs sprouting from a single skeletal muscle fibre cultured as reported above, fixed and immunostained with antibodies against laminin 2α (green) and MyoD (red). Arrows indicate the nuclear localization of MyoD. Quantitative analysis of the number of Pax-7$^+$ cells in the different experimental conditions and the densitometric analyses of the intensity of MyoD fluorescence signal, performed on digitized images are reported in the histograms. (B) Phase contrast microscopy analysis of myotube formation after 48 h culture of SCs in the indicated experimental conditions; the quantitative analysis of the myotube number is reported in the corresponding histogram. Data are representative of at least three independent experiments with similar results. Significance of differences was evaluated by Student's *t* test: $^*p<0.05$ *vs* control in (A) and *vs* SCs control in (B).

Fig. 5 – MMP-2 and MMP-9 and TIMP-1 and TIMP-2 expression in Pax-7 positive satellite cells exposed to factors secreted by MSCs. Satellite cells (SCs) isolated from single skeletal muscle fibre, at the first passage (P1), were cultured in growth medium (SCs control) or exposed to MSC-conditioned medium (SCs+MSC-CM) for 24 h. (A) Western blotting analysis of MMP-2 and MMP-9 expression. The densitometric analysis of the bands normalized to α-tubulin, is reported in the histogram. (B) Representative immunofluorescence confocal images of SCs in the indicated experimental conditions, immunostained with antibodies against Pax-7(red), MMP-2 (green), MMP-9 (cyan), TIMP-1 (green) and TIMP-2 (green). Densitometric analyses of the intensity of the fluorescence signals of MMP-2, MMP-9, TIMP-1 and TIMP-2 performed on digitized images, are reported in the histograms. Data are representative of at least three independent experiments with similar results. Significance of differences was evaluated by Student's *t* test: $^*p<0.05$ *vs* SCs control; $^\circ p<0.05$ *vs* MMP-2.

study that MSCs, which are emerging as good candidates for cell-based therapy to improve skeletal muscle repair/regeneration [17–19], secrete paracrine factors capable of up-regulating MMPs in both skeletal muscle specific stem cells, myoblasts and satellite cells, and skeletal fibroblasts, thus providing clues for additional therapeutic options to counteract fibrosis in diseased skeletal muscle. Indeed, when compared to controls, C2C12 myoblasts and satellite cells co-cultured with MSCs or exposed to MSC-CM



exhibited increased MMP-2 and MMP-9 expression and activity associated with a down-regulation of MMP inhibitors, TIMP-1 and TIMP-2. These data are consistent with our previous reports showing that C2C12 myoblasts and myofibre-associated satellite cells express MMP-2 and MMP-9 [14,31] and that MSCs produce and release a wide variety of cytokines and growth factors, including interleukin (IL)-1, basal fibroblast growth factor (bFGF) and VEGF [20,28], which are considered highly potent inducers of MMP expression in many cell types [57]. In particular, it has been recently demonstrated that injection of VEGF into wounded skin increases neo-epidermal thickness and enhances re-epithelialization through the up-regulation of MMP-2 and MMP-9 expression [58] and preliminary data from our laboratory have indicated that the selective pharmacological VEGFR inhibition, using KRN633, markedly attenuates the expression of MMP-2 in C2C12 cells [personal communication].

We also showed that MSC-CM promoted myoblast and satellite cell motility. In particular, using a scrape migratory assay, we demonstrated that MSC-CM-mediated myoblast motility was largely prevented by the incubation with a potent MMP inhibitor, SB-3CT. The inhibition of migration correlated with changes in cell morphology and in the cytoskeletal organization. Indeed, the typical actin motile structures, namely microspikes and ruffles/lamellipodia, observed at the migratory front were largely absent in the myoblasts pretreated with the MMP inhibitor prior the exposure to MSC-CM. In view of a previous study showing that MMP-2 physical interact with integrin and the actin cytoskeleton in glial cells [59], it is possible to suggest that the increased MMP expression observed in myoblasts in response to paracrine factors from MSCs may influence myoblast cells migration not only through the increased pericellular proteolysis but also through the stimulation of functional interactions with the actin motile structures. This could also explain some of our data concerning the prevalent cytoskeletal distribution of MMP-2 and MMP-9 in the myoblastic C2C12 cells. On the other hand, the intracellular activity of MMPs is in line with the newly emerging evidence that these proteases, in particular MMP-2, beside their role as secreted proteins on ECM, can also rapidly act on several specific substrates inside the cells [60]. Moreover, the nuclear localization of MMPs in different cell types involves a novel biological action of these MMPs, consisting in a direct involvement in the regulation of gene transcription and expression [46,60–62].

Another interesting observation of the present study was the ability of MSC-CM to negatively interfere with myofibroblast differentiation of skeletal fibroblasts in primary cultures. Since myofibroblasts are believed to be the major contributors to tissue scarring, such findings provide further evidence for an anti-fibrotic effect of the conditioned media harvested from MSCs. Indeed, we have found that MSC-CM markedly attenuated the tendency of skeletal fibroblast to produce α-sma and type-I collagen. These data are consistent with those conducted in another study where vascular smooth muscle cells transplanted into the infarcted rat heart decreased myofibroblast activation and the subsequent maladaptive structural remodeling through paracrine mechanisms [63]. Interestingly, the inhibition of fibroblast-myofibroblast transition observed after the exposure to MSC-CM was significantly impaired when the cells were pre-treated with SB-3CT, suggesting the intriguing hypothesis that the cultured medium could suppress this phenomenon through putative MMP-dependent mechanisms. In support of this hypothesis, there are recent data that suppression of MMP-2 with TIMP-1 blocks hepatocyte growth factor (HGF)-decrease, α-sma and type-III collagen expression in transforming growth factor (TGF)-β-treated Achilles tendon fibroblasts [64]. The mechanisms whereby MMPs negatively influence myofibroblast activation and fibrosis are completely unknown. Efforts will continue in our laboratory to address this point and, in particular, to reveal any potential involvement of MMPs in the synthesis and secretion of profibrotic factors, such as TGF-β by the skeletal fibroblasts.

On the basis of the results of the present study it would be reasonable to hypothesize that the beneficial effects of MSC therapy for skeletal muscle repair/regeneration may also involve the ability of the engrafted cells to stimulate ECM remodeling and myoblast migration, as well as to attenuate myofibroblast formation and the subsequent excessive collagen production. In so doing, the engrafted cells and their released factors modify the local microenvironment and disrupt the mechanical barriers against the recruitment of the endogenous stem cells at the sites of muscle injury.To the best of our knowledge, this is the first experimental study to report the potential of MSC-derived paracrine factors to assist muscle remodelling through the upregulation of MMPs in different cell types.

We believe that the unique value of MSC therapy over the administration into the damaged muscle of single molecules, such

Fig. 7 – MMP-2 and MMP-9 expression/activity and TIMP-1 and TIMP-2 expression in skeletal muscle fibroblasts. Primary skeletal muscle fibroblasts (SkmFs) were cultured for 24 h in single culture in low serum culture medium (SkmFs control), co-coltured with GFP-MSCs (SkmFs+GFP-MSCs) or exposed to factors secreted by MSCs in the absence (SkmFs+MSC-CM) or presence of 5 or 10 μM SB-3CT (SB). (A) Representative superimposed DIC and confocal immunofluorescence images of SkmFs immunostained with antibodies against vimentin (green) and von Willebrand factor (green). Nuclei are counterstained in red with propidium iodide (PI). (B) Western blotting analysis of MMP-2 and MMP-9 expression in SkmFs in the indicated experimental conditions. The densitometric analysis of the bands normalized to α-tubulin is reported in the histogram. Data are representative of at least three independent experiments with similar results. (C) Confocal immunofluorescence analysis of MMP-2 (green), MMP-9 (cyan), TIMP-1 (green) and TIMP-2 (green) expression in the indicated experimental conditions; GFP fluorescent signal is shown in red pseudocolor. (D) Densitometric analyses of the intensity of the fluorescence signals of each specific marker performed on digitized images. (E) Gelatinase assay. SkmFs were seeded on a fluorescein-labeled gelatin substrate (DQ™ gelatin) and cultured in the indicated experimental conditions. Spectrophotometrical quantification of the DQ™ gelatin fluorescence intensity revealed after proteolytic digestion of the gelatin by MMP gelatinases. MSC-CM refers to the sole MSC-CM added to the DQ™gelatin substrate. Significance of differences in B was evaluated by Student's *t* test: *$p<0.05$ *vs* SkmFs control; in (D), it was evaluated by one-way ANOVA and Newman–Keuls multiple comparison test: *$p<0.05$ *vs* SkmFs control, °$p<0.05$ *vs* SkmFs+GFP-MSCs, in E it was evaluated by one-way ANOVA and Newman–Keuls multiple comparison test: #$p<0.05$ *vs* MSC-CM, §$p<0.05$ *vs* SkmFs control; **$p<0.05$ *vs* SkmFs+MSC-CM.

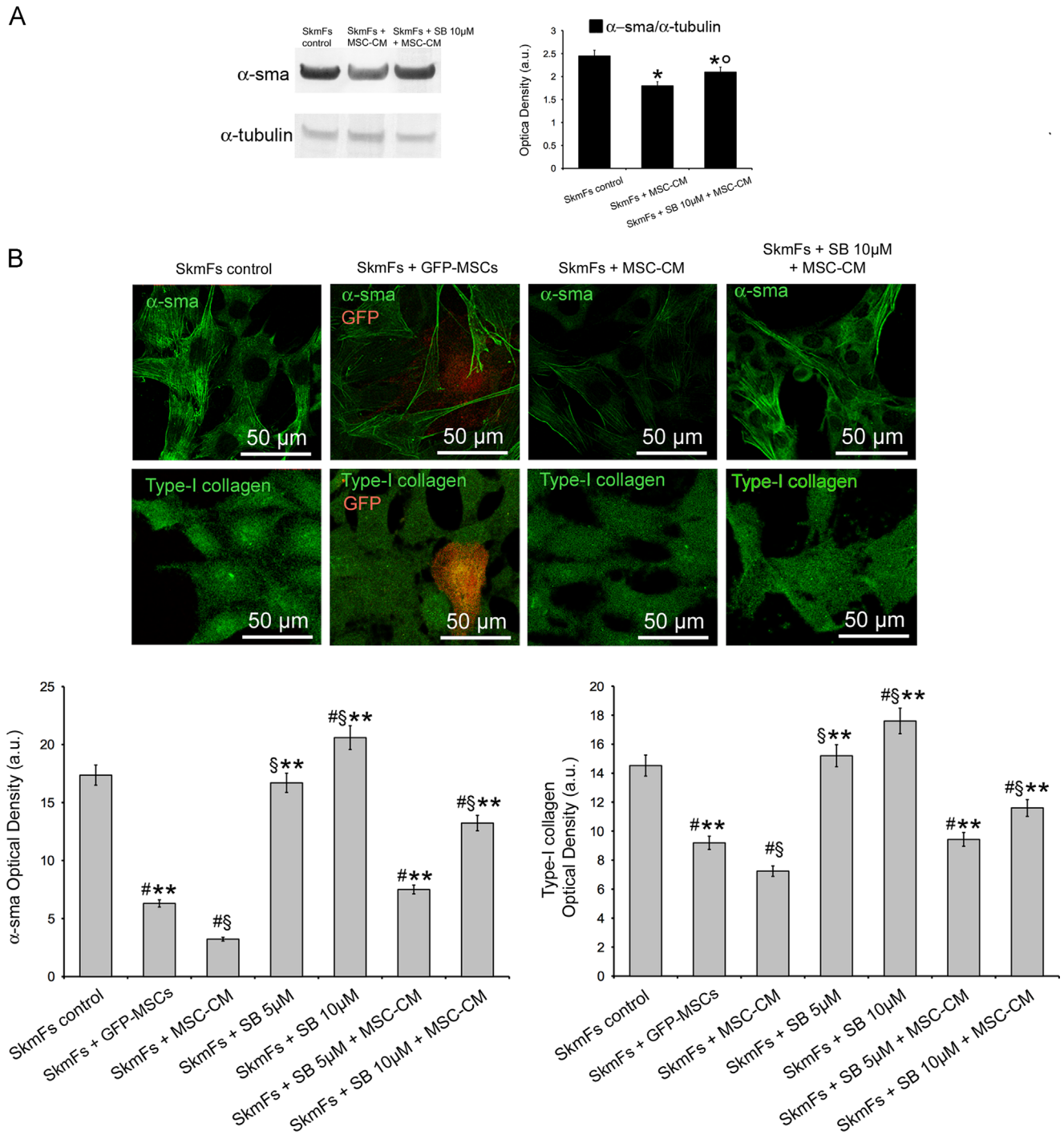


Fig. 8 – Fibroblast-myofibroblast transition after exposure to MSC-conditioned medium and inhibition of MMP activity. Primary skeletal muscle fibroblasts (SkmFs) were cultured for 24 h in single culture in low serum culture medium (SkmFs control), co-coltured with GFP-MSCs (SkmFs+GFP-MSCs) or exposed to factors secreted by MSCs in the absence (SkmFs+MSC-CM) or presence of 5 or 10 μM SB-3CT (SB). (A) Western blotting analysis of α-sma expression in SkmFs in the indicated experimental conditions. The densitometric analysis of the bands normalized to α-tubulin is reported in the histogram. Data are representative of at least three independent experiments with similar results. (B) Confocal immunofluorescence analysis of α-sma and type-I collagen expression (green) in the indicated experimental conditions; GFP fluorescent signal is shown in red pseudocolor. Densitometric analyses of the intensity of the fluorescence signals of each specific marker performed on digitized images are reported in the histograms. Significance of differences was evaluated by one-way ANOVA and Newman–Keuls multiple comparison test. In (A) $^{*}p<0.05$ *vs* SkmFs control, $^{\circ}p<0.05$ *vs* SkmFs+MSC-CM; in (B) $^{\#}p<0.05$ *vs* SkmFs control, $^{\S}p<0.05$ *vs* SkmFs+GFP-MSCs, $^{**}p<0.01$ *vs* SkmFs+MSC-CM.

as MMPs and/or their inhibitors or anti-fibrotic factors, which suffer of rapid clearance and relatively short half-life [7], may reside in the possibility of the engrafted cells to release constant levels of paracrine signals that can act synergistically to stimulate the release of MMPs in the contest of the regenerating tissue to achieve more sustained therapeutic effects.

In conclusion, our data contribute to extend the therapeutic potential of MSCs for the treatment of diseased or injured skeletal muscle and open new prospective for understanding the mechanisms of action of MSCs in muscle healing. Our findings provide circumstantial evidence that these cells exert potent anti-fibrotic effects at least in part through the regulation of MMP/TIMP expression on different muscle cells and can be exploited to augment the beneficial effects of cell transplantation and improve and sustain their engraftment into the damaged muscle.

Conflict of interest statement

None.

Acknowledgments

This study was supported by research founding from MIUR (Ministero dell'Istruzione dell'Università e della Ricerca)- Italy to C.S, D.N., L.F. and S.Z-O. and by a research grant from the Ente Cassa di Risparmio di Firenze (Florence, Italy) to L.F.

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