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Supplemental Information

In Vivo Therapeutic Potential of Mesenchymal Stromal Cells Depends on the Source and the Isolation Procedure

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SUPPLEMENTAL FIGURES AND LEGENDS

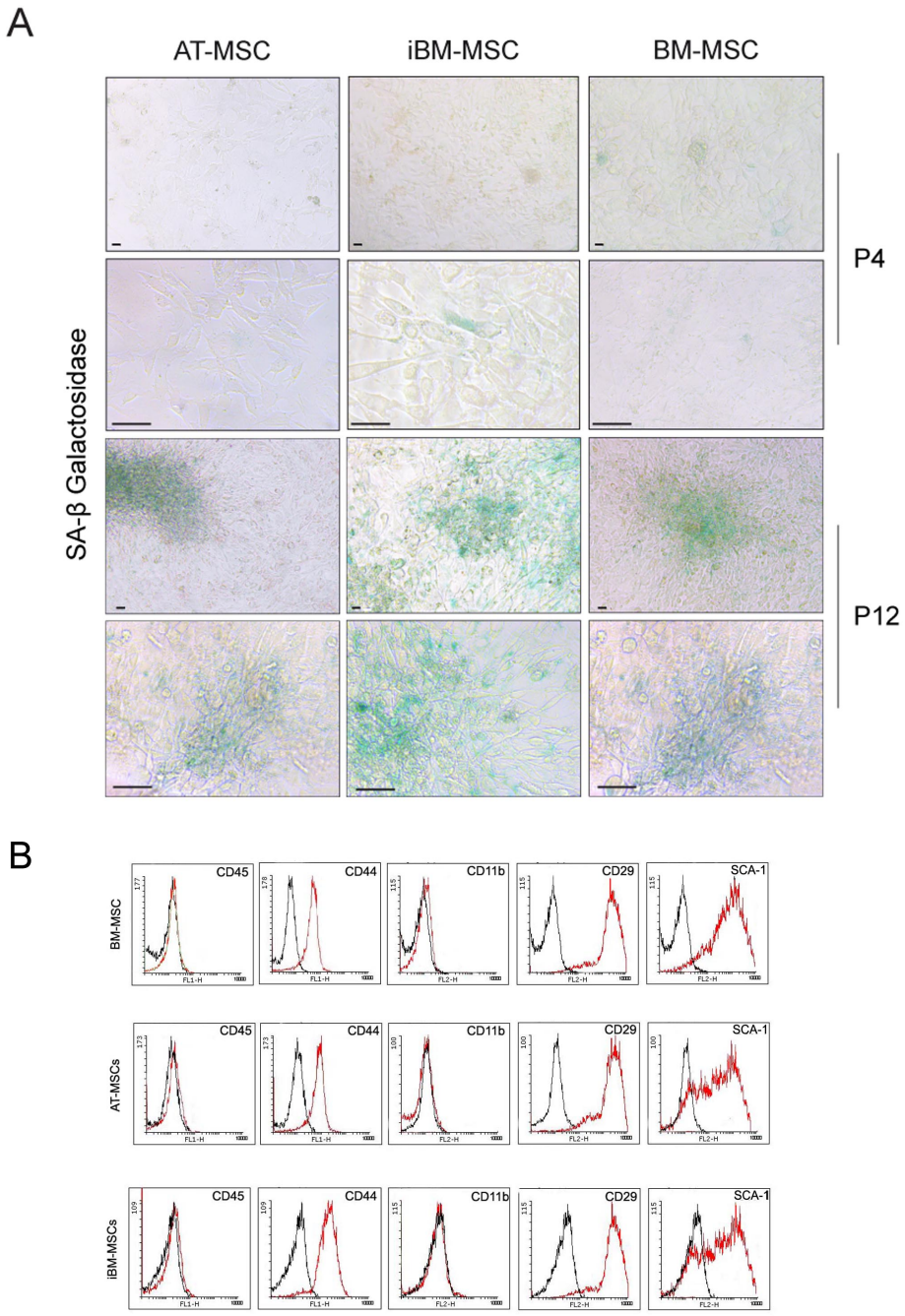

Figure S1

<u>Figure S1.</u> Characterization of cellular senescence and surface marker expression for the three MSC populations.

Related to Figure 1.

A. Representative images of senescence-associated β-galactosidase (SA-βgal) expression detected in MSC cultures, at early (4) and late (12) passages. Scale bar 50 μm for lower magnification and 10 μm for higher magnification.

B. Flow cytometry plots for the surface antigens commonly recognized as MSC markers. The black line refers to isotype control antibody, whereas the red line corresponds to the three MSC populations stained using the indicated antibodies.

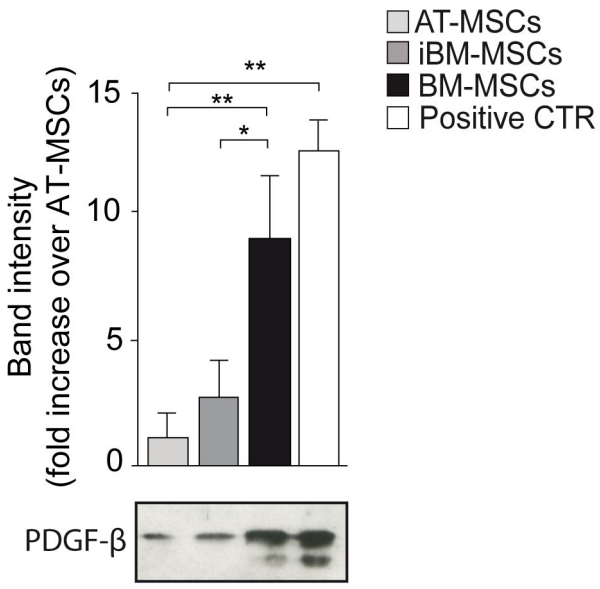

Figure S2

<u>Figure S2</u>. BM-MSCs secrete high levels of PDGF-β.

Related to figure 3.

Analysis of PDGF-β secretion by MSCs. The same number of AT-, iBM- and BM-MSCs was plated and kept in culture for 24 hours in serum-free medium. The presence of PDGF-β was detected by Western Blotting. Supernatant from PDGF-β-transfected cells was used as a positive control. Data (n=3 biological replicates) were normalized over AT-MSCs and shown as mean ± SEM (**P*< 0,05; ***P*<0,01).

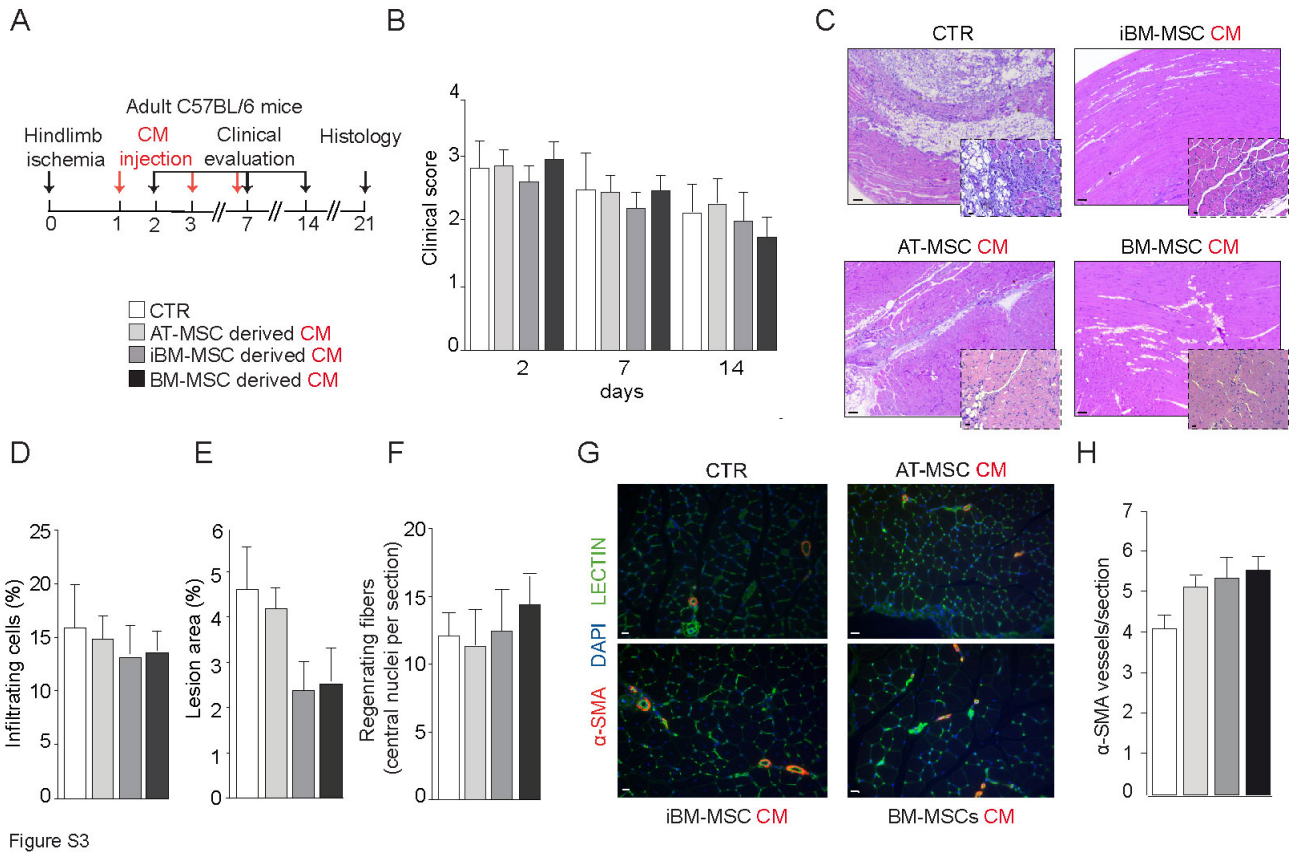

Figure S3. MSC-derived Conditioned Medium (CM) partially recapitulates the therapeutic effect of MSC administration after ischemia.

Related to Figure 3.

A. CLI experimental flow chart. Mice received concentrated CM derived from AT-, BM- or iBM- MSCs at passage 4 or medium without serum (n=6 animals for each group) at days 1, 3 and 7 after resection of the femoral artery.

B. Limb function evaluation at days 2, 7 and 21 after CLI according to the criteria described in **Table S2**.

C. Representative hematoxylin-eosin staining of ischemic muscles of untreated and treated animals at day 21. Scale bar 100 μm for lower magnification and 10 μm for higher magnification.

D. Percentage of infiltrating cells at the site of ischemia. Data are shown as mean ± SEM.

E. Percentage of muscle affected by ischemic damage. Data are shown as mean ±

SEM.

F. Quantification of central nuclei as a hallmark of muscle regeneration. Data are shown as mean ± SEM.

G. Representative immunostaining for vascular structures in ischemic muscles treated with either serum-free medium or CM derived from, AT-, BM- or iBM-MSCs at day 21. α-SMA red; LECTIN, green; DAPI, blue; scale bar 50 μm.

H. Quantification of the number of α-SMA positive vessels. The graph shows mean ± SEM.

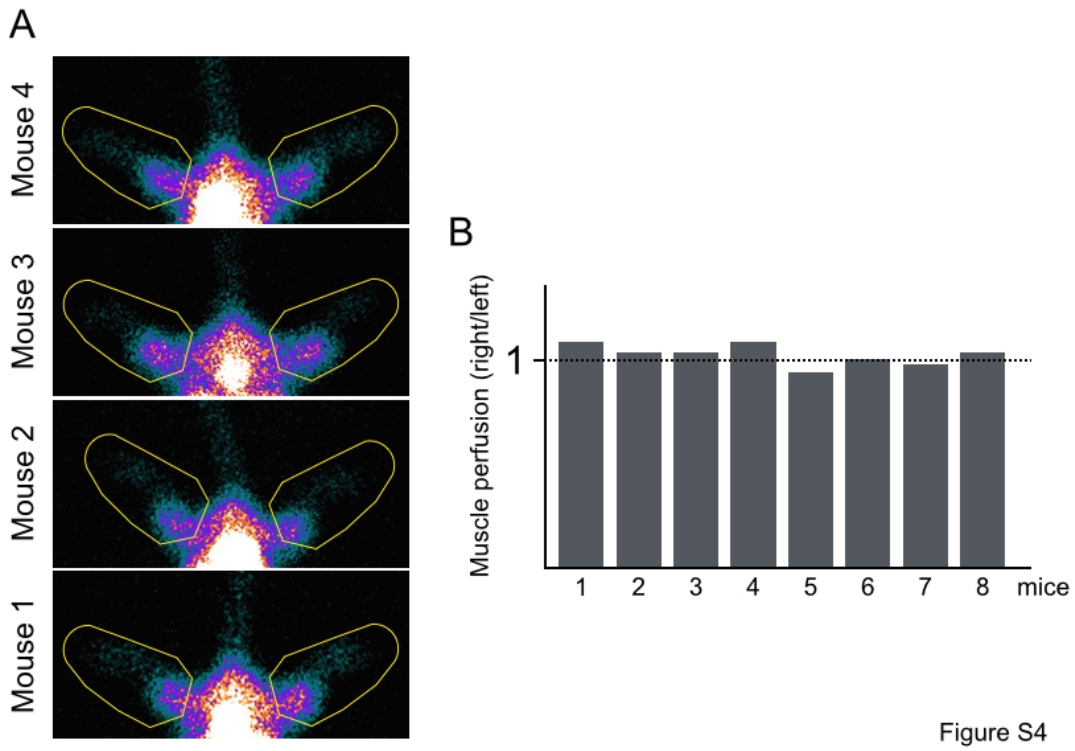

Figure S4. Planar Scintigraphy at baseline reveals equal perfusion of the two hind limbs

Related to Figure 4.

A. Representative images of planar scintigraphy showing the ROI (yellow line), used to quantify muscle perfusion on both hind limbs in 4 animals before surgery.

B. Quantification of muscle perfusion, expressed as the right to left ratio of counts within the ROI in 8 randomly selected animals before surgery.

SUPPLEMENTAL TABLES

Table S1. Surface antigen expression by cultured MSCs from different sources analyzed by flow cytometry.

Related to Figure 1.

For clarity, a symbol related to the percentage of positive cells was assigned to each marker: - < 10%; + 10-30%; ++ > 30%.

Surface antigen	AT-MSCs	BM-iMSCs	BM-MSCs
CD105	+	+	+
CD11b	-	-	-
CD29	++	++	++
CD31	-	-	-
CD34	-	-	-
CD44	++	++	++
CD45	-	-	-
CD90	+	-	+
C-KIT	+	+	+
CXCR4	+	+	+
FLK1	-	-	-
GR-1	-	-	-
SCA-1	++	++	++
TER119	+	+	+
TIE-2	+	+	+

Table S2. Score assignment criteria for evaluation of ischemic damage.
Related to Figure 2.

Score	Clinical evaluation of left hind limb
0	No differences from contralateral hind limb
1	Mild hypomobility and discoloration
2	Moderate hypomobility and discoloration
3	Severe hypomobility and distal necrosis
4	Paw dragging and massive gangrene

Table S3. List of genes analyzed for expression by real-time, quantitative PCR.

Related to Figure 3.

Gene acronym	Gene name	Ref. Seq	Real time PCR assay
Ang-1	angiopoietin 1	NM_009640.3	Mm00456503_m1
Ang-2	angiopoietin 2	NM_007426.3	Mm00545822_m1
Ccl2	chemokine (C-C motif) ligand 2	NM_011333.3	Mm00441242_m1
Ccl5	chemokine (C-C motif) ligand 5	NM_013653.3	Mm01302427_m1
Cxcr4	chemokine (C-X-C motif) receptor 4	NM_009911.3	Mm01292123_m1
Cxcl9	chemokine (C-C motif) ligand 4	NM_013652.2	Mm00443111_m1
Cxcl10	chemokine (C-X-C motif) ligand 10	NM_021274.1	Mm00445235_m1
Flt-1	FMS-like tyrosine kinase 1	NM_010228.3	Mm00438980_m1
Flk-1	kinase insert domain protein receptor	NM_010612.2	Mm00440099_m1
Mmp9	matrix metallopeptidase 9	NM_013599.2	Mm00442991_m1
Nrp-1	neuropilin 1	NM_008737.2	Mm00435372_m1
Pdgf-β	platelet derived growth factor, B polypeptide	NM_011057.3	Mm00440678_m1
Plx1	plexin A1	NM_008881.2	Mm00501110_m1
Rgs-5	regulator of G-protein signaling 5	NM_009063.3	Mm00501393_m1
Sdf-1	stromal cell-derived factor 1	NM_013655.3	Mm00445552_m1
Tgf-β	transforming growth factor, beta	NM_011577.1	Mm00441724_m1
uPa	plasminogen activator, urokinase	NM_008873.2	Mm01274460_g1
Vegf-a	vascular endothelial growth factor A	NM_009505.4	Mm00437304_m1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	NM_008084.2	Mm99999915_g1

All the amplifications were performed using pre-developed assays (Applied Biosystems).

The housekeeping gene *Gapdh* was used to normalize the results.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Murine mesenchymal stromal cells isolation and culture

Bone marrow

Briefly, by flushing the tibia and femur bone cavity with Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) supplemented with penicillin/streptomycin (P/S, 100 U/mL and 100 g/mL), a cell suspension was obtained and filtered through a 70 μm filter mesh to remove any bone spicule or muscle and cell clump. MSCs were then isolated according to two different protocols.

BM-MSCs were isolated using an adapted protocol, previously published (Soleimani and Nadri, 2009). This protocol is based on MSC physical propensity to adhere to plastic substrates and on frequent medium changes to avoid contamination by hematopoietic cells. BM-MSCs were cultured in complete medium (DMEM-HG supplemented with 10% heat-inactivated fetal bovine serum (FBS) and P/S) and non-adherent cells were removed after 3 hours, followed by fresh complete medium replacement. This step was repeated every 12 hours for 3 days. Cells reached 90% confluence in approximately 2 weeks and were lifted by incubation in 0.5 ml of 0.25% trypsin/1 mM EDTA for 2 minutes at room temperature. Lifted cells were cultured in 25-cm^2 flask until confluence at 37°C in a 5% CO_2 humidified incubator, changing the medium every 3–4 days.

iBM-MSCs isolation was based on a density gradient, followed by immunodepletion of monocytes and granulocytes (Sung et al., 2008). After bone flushing, cells were suspended in equal amount of DMEM and Hank's Balance Salt Solution (HBSS) and all mononuclear cells (MNCs) were isolated by density-gradient centrifugation using Histopaque-1077 (Sigma Chemical Co.), according to the manufacturer protocol. MNCs were then immunodepleted of monocytes and granulocytes using anti-CD11b-coupled micromagnetic beads (Miltenyi Biotec.). The residual cells were cultured in complete medium, as described above. After removal of non-adherent cells after 72–96 hours,

adherent cells were harvested by trypsinization (0.05% trypsin-EDTA) when reaching 90% confluence, and then replated. The medium was changed every 3–4 days.

Adipose Tissue

Subcutaneous adipose tissue was cut into fine pieces and digested with 1 mg/mL collagenase IA (Sigma) for 90 minutes at 37°C while shaking. The released cells were centrifuged for 15 minutes at 400g, washed with PBS twice and filtered through a 70 µm mesh. To separate MSCs from adipocytes, the cell suspension was subjected to two additional centrifugation steps at 800g. Pelleted MSCs were resuspended in lysis buffer (BD) to lyse and remove red blood cells. The residual cells were eventually cultured as described for BM-derived cells.

At least 3 independent isolations were performed for each isolation protocol.

Animal care and hind-limb ischemia

Animal care and treatment were conducted in conformity with institutional guidelines, in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12th, 1987 and UE2010763) after institutional review board approval. Mice (6-week-old female C57/BL6) were anesthetized using xylazine (40 mg/kg) and ketamine (100 mg/kg), prior to ligation and removal of the entire left femoral artery. The following day, mice were randomly divided into 4 groups (n>10) and injected intramuscularly with AT-, BM-, iBM-MSCs or medium as control. Each animal received $5x10^4$ (passage 3-5) cells in its left tibialis anterior muscle and $1,5x10^5$ cells in its left gastrocnemious muscle (final volume: 50 µl and 150 µl, respectively). Mice were repeatedly monitored for limb function, according to the parameters described in **Table S2**. At the end of the experiments (day 21), the injected muscles were harvested, embedded in paraffin and processed for histological analysis. The tibialis muscles were analyzed on transversal sections, while the gastrocnemious

muscles on longitudinal sections.

For cell fate and retention experiments, MSCs were labeled with DiI dye (Invitrogen) and administered as previously described into the left tibialis anterior and gastrocnemious muscles the day after CLI (n= 6 per group).

Planar Scintigraphy

Planar Scintigraphy was performed prior to surgery (baseline, shown for 8 representative animals in **Figure S4**), on day 1 (immediately before MSC injection), as well as on days 7 and 14 (n=8 per group). Functional images of the mouse hind limbs were acquired after the injection of 37 MBq (1 µL) of 99mTc tetrofosmin (Myoview®, GE Healthcare) into the jugular vein (small pinhole collimator hole: 4 mm; frame-acquisition time 10 min; zoom acquisition 3.2). Quantitative analysis was made by manually drawing an oval region of interest (ROI) first on the ischemic hind limb and then symmetrically applying the same ROI to the contralateral limb. ROI was demarcated paying attention to avoid abdominal radioactivity and to include limb extremities. Perfusion data at each time point were expressed as a right/left ratio to overcome inter-animal variability and eventually normalized to the perfusion measured at day1 to overcome possible differences due to the severity of muscle ischemia.

Flow cytometry

Cells surface antigens were analyzed by flow cytometry using a BD FACSCalibur. MSCs ($1x10^5$ cells at passage 3) were incubated on ice for 30 minutes in PBS, 2% BSA and stained with fluorescein isothiocyanate- (FITC-) or phycoerythrin (PE-) conjugated monoclonal antibodies against mouse CD44, CD45, CD31, CD29, CD11b, CD105, CD34, CD90, CD117 (C-KIT), TER-119, stem cell antigen (SCA)-1, CXCR4, GR-1, FLK1, TIE2 (all from BD), or isotype-matched mouse monoclonal antibodies (BD) as controls. Data are

summarized in **Table S1 and Figure S1B**.

Colony Forming Unit assay (CFU) and cell proliferation assay

For the proliferation assay murine AT-, iBM-, BM-MSCs ($1x10^5$ cells at passage 2) were plated in 6-multiwell plates and counted daily for four days. For CFU assay, MSCs (250 cells at passage 2) were plated in a 6-multiwell plate; after 10 days of culture, cells were stained with Crystal Violet and the number of colonies was counted manually. The experiments were performed in triplicate and results expressed as mean±SEM.

Differentiation assays

Differentiation was performed on cells at passage 3 using Adipolife, OsteoLife and ChondroLife complete medium, respectively (all from LifeLine Cell Technologies). Oil Red, Alizarin Red, and Alcian Blue (LifeLine Cell Technologies) staining for adipocytes, osteocytes, and chondrocytes, were performed according to the manufacturer protocols.

Histology and immunofluorescence

Cultured MSCs were immunostained for CD44 (Abcam, 1:100) and CD105 (R&D system, 1:100), followed by goat anti-rabbit or donkey anti-goat AlexaFluor 594 (Molecular Probes, 1:1000). All images were acquired using a fluorescence microscope (Leica Microsystems), equipped with a charge-coupled device camera. In all experiments, exposure and picture processing were applied equally to sample sections and controls (isotype or primary antibody present).

Muscle sections were stained with hematoxylin-eosin to perform morphometric analysis of tissue damage, cell infiltration (on longitudinal section), and fiber regeneration (on transversal section) by acquiring at least 10 images per mouse and using NIH ImageJ software for data quantification (data are expressed as mean±SEM).

To analyze tissue vascularization, sections were stained with Cy3-conjugated mouse monoclonal antibody anti-α-SMA (Sigma, 1:200), FITC-conjugated LECTIN from Triticum vulgaris (Sigma, 1:200) for 2 hours. At least 10 images per muscle were acquired and the number of arteries was expressed as the number of α-SMA-positive vessels (mean±SEM). To track cell engraftment *in vivo,* MSCs were labeled with DiI (Invitrogen) prior to injection. Injected muscles were snap frozen and cryosections were stained for 2 hours at RT with FITC-LECTIN. Quantification of retained cells was performed using the ImageJ software (9 slices every 50 μm per muscle, 6 fields per section). To track cell differentiation in vivo, muscles at 21 days were stained with rat polyclonal anti-CD31 (1:100, BD Pharmingen) or Cy3-conjugated α-SMA (1:200, Sigma) antibodies, followed by goat anti-mouse AlexaFluor 488 (1:500, Molecular Probe) or goat anti-rat AlexaFluor 488 antibody (1:500, Invitrogen), respectively.

Real-Time PCR

Total RNA was extracted from MSCs isolated according to the three experimental procedures (n=3 each) using TRIzol reagent (Invitrogen) according to manufacturer instructions, treated with DNase I (Roche) and reverse transcribed using hexameric random primers. The cDNA was then used as a template for real-time PCR amplification to detect the expression levels of the murine genes listed in **Table S3.** The housekeeping gene *Gapdh* was used to normalize the results. All the amplifications were performed on a Bio-Rad Real-time thermal cycler CFX96 using TaqMan probes (Applied Biosystem).

Migration assay

Migration assays were performed using 8 μm pore Transwell permeable supports (Costar, Corning Incorporated). MSCs (isolated from at least 3 animals per MSC type, 10^5 cells per animal) were seeded in the lower chamber, whereas mouse primary aortic smooth muscle

cells (C57-6080; CellBiologics; 1×10^4 cells) were seeded in serum-free medium in the upper chamber. After 12 hours, the upper side of the filters was carefully washed with PBS, and migrated cells on the lower surface of the membrane were fixed in PFA and stained with DAPI (Vector Lab.). Relative areas occupied by migrated cells were quantified using Image J (National Institutes of Health). Experiments were performed in triplicate by quantifying the number of migrated cells on membrane images acquired by ImageXpress Micro automated high-content screening fluorescence microscope. Data are presented as mean±SEM.

Apoptosis assay

MSC apoptosis was induced in 6-multiwell plates adding 500 μM of hydrogen peroxide (H_2O_2). The Annexin V-FITC Apoptosis Detection Kit (Roche) was used to quantify the number of apoptotic cells by flow cytometry, according to manufacturer instructions. Apoptosis induced by doxorubicin (2 μM for 12 hours) was evaluated using Caspase-Glo 3/7 Assay Systems (Promega) in 96-multiwell plates, according to manufacturer instructions and analyzed by the EnVision multilabel reader (Perkin Elmer). All experiments were performed in triplicate and data, normalized over untreated cells, are presented as mean±SEM.

Senescence assay

Senescence associated-β galactosidase (SA- β gal) was evaluated on cells at passages 4 and 12, kept in culture for 5 days. Cells were washed with PBS, fixed for 10 minutes in 4% PFA, washed in PBS and incubated at 37°C with senescence X gal stain solution (1 mg X-galactose; 40 mM citric acid/sodium phosphate at pH 6; 5mM potassium ferricyanide; 150mM NaCl; 2mM $MgCl_2$). Images of stained cells were acquired after 12 hours.

Conditioned medium preparation

MSCs ($1x10^6$ cells at passage 4) were plated in 6 cm^2 dishes and incubated in complete medium for 1 day. Cells were then washed with PBS and the medium was replaced with 1 ml of serum free medium. Media were collected after 24 hours, concentrated using the Amicon Ultra 0,5 Centrifugal Devices (cut-off 3 kDa; Millipore) and filtered through a 20 µm filter. The concentrated conditioned media (CM) were frozen and stored at -80°C. Three doses of CM corresponding to $2x10^5$ MSCs were injected in the tibialis anterior and in the gastrocnemius muscles of C57/BL6 mice at day 1, 3, 7 after surgery (n=6 animals per group). Animals were followed for 21 days for functional and histological evaluation.

Western Blot

To quantify the levels of PDGF-β secreted by the three MSC populations, CM was collected from the same number of AT-, iBM- and BM-MSCs. Samples were resolved by 10% SDS-PAGE and transferred to PVDF membranes (GE Healthcare). Immunoblots were blocked in 5% bovine serum albumin in TBS-Tween (50 mM Tris-HCl, pH 7.4; 200 mM NaCl; and 0.1% Tween 20), incubated O/N at 4^oC with primary rabbit polyclonal anti–PDGF-β antibody (sc-7878 Santa Cruz) and then with anti-rabbit HPR-conjugated secondary antibodies for 1 hour at room temperature. Proteins were detected by enhanced chemiluminescence (GE Healthcare). CM obtained after transfection of a *Pdgf-β* plasmid was used a positive control for the antibody. Band intensity was analyzed using the ImageJ software.

SUPPLEMENTAL BIBLIOGRAPHY

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