Supplementary Material to Rossi et al. "Co-injection of mesenchymal stem cells with endothelial progenitor cells accelerates muscle recovery in hind limb ischemia by an endoglin-dependent mechanism" (https://doi.org/10.1160TH17-01-0007)

Suppl. Figure 1. MSCs characterization by flow cytometry

Suppl. Figure 2. ECFCs endoglin inhibition by siRNA, checked by flow cytometry

ECFCs silencing results to be more efficient at 48-72h. The graph is the medium of 3 assays.
c-siRNA, control-siRNA; e-siRNA, endoglin-siRNA.
Suppl. Figure 3. ECFCs endoglin inhibition by siRNA, checked by immunofluorescence and western blot

A - Immunofluorescence on ECFC (green endoglin, red vWF, blue nuclei). 40X magnification : scale bar 50µm

B, Western Blot-reducing condition, comparison between ECFC control and ECFC where endoglin was inhibited by siRNA. Endoglin monomer is 90KDa.
For western blot, cells (controls and endoglin siRNA) were lysed at 4 °C with RIPA Lysis Buffer. Total protein quantification was performed using BCA kit (Thermo fisher Scientific). A 4-12% gradient gel was used under reducing conditions followed by transfer to nitrocellulose membranes according to the technical bulletin of the manufacturer (Thermo fisher Scientific). Proteins were revealed by incubation with human P4A4 antibody (Hybridoma) followed by incubation with Goat anti-mouse Dylight 680 according to the manufacturer's instructions. Infrared fluorescence detection was performed with the Odyssey Infrared Imaging System (LI-COR Biosciences).
Suppl. Figure 4. ECFC and MSC injection in hind limb ischemia have a homing in ischemic leg

B- Representative photomicrographs of fluorescence observed in mice's eye after i.v. injection stained with Visen technology after iv injection and 48 hours after injection.

C- Analysis by flow cytometry ECFC and MSC previously labelled with vivo tag 680 and 750 in non-ischemic and ischemic muscle (HoWangYin et al. 2014; Smadja et al. 2014). ECFC are labelled with Vivotag 750 dye and appear in APC-Cy7. MSC are labelled with Vivotag 680 dye and appear in APC.
Suppl. Figure 5. Analysis of necrosis according to (Willett et al. 1987)

20X magnification: scale bar 100 µm

A- Normal / undamaged myofibers: Normal myofibers have peripheral nuclei, intact sarcolemma and non-fragmented sarcoplasm. In young mice these myofibers represent areas of muscle that have not undergone the process of necrosis and regeneration. In adult mice these myofibers represent areas of muscle that have either never undergone necrosis or have previously undergone necrosis and regeneration with long enough time for the myonuclei to have moved to the periphery.

B- Necrotic myofibers: Necrotic muscle is identified by the presence of infiltrating inflammatory cells (basophilic staining) and/or hypercontracted myofibers and degenerating myofibers with fragmented sarcoplasm. Specifically, in this example we have hind limb ischemia, PBS injection (negative control) and analysis after 14 days. Arrows indicate collapse and histiocytes/inflammation cells infiltration.

C- Regenerating (recently necrotic): Regenerating muscle is identified by activated myoblasts and, small basophilic myotubes. These myotubes subsequently mature into plump myofibers with central nuclei (regenerated myofibers, arrow). Specifically, in this example we have hind limb ischemia, ECFC injection (positive control) and analysis after 14 days. Normally after 20 days this regeneration is completed. Presence of myofibers depends on how faster is the recovering process. Sometimes at 14 days the tissue is completely recovered.
Suppl. Figure 6. MSCs transdifferentiation assays

A, B. MSCs after 7 days of culture with smGM2 or EGM2 media respectively and 2 weeks of treatment with adipogenic/osteogenic differentiation media were stained for adipogenic differentiation (Nile Red, NR) or osteogenic differentiation (Phosphatase Alcaline, ALP / Alizarine Red, AR) was performed. EGM2 seems to induce the fastest transdifferentiation of MSCs in adipocytes and osteocytes.

10X magnification: scale bar 200 µm
Suppl. Figure 6. MSCs transdifferentiation assays

**C,D** Comparison between ECFCs endoglin-siRNA and ECFCs control-siRNA in the same conditions. No difference in terms of trasdifferentiation between endoglin-silenced cells and controls (scramble) was observed. Pictures are taken at 20x magnification.

10X magnification: scale bar 200 µm

Suppl. References