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Supplemental Information

Coupling between Myogenesis and Angiogenesis during Skeletal Muscle Regeneration Is Stimulated by Restorative Macrophages

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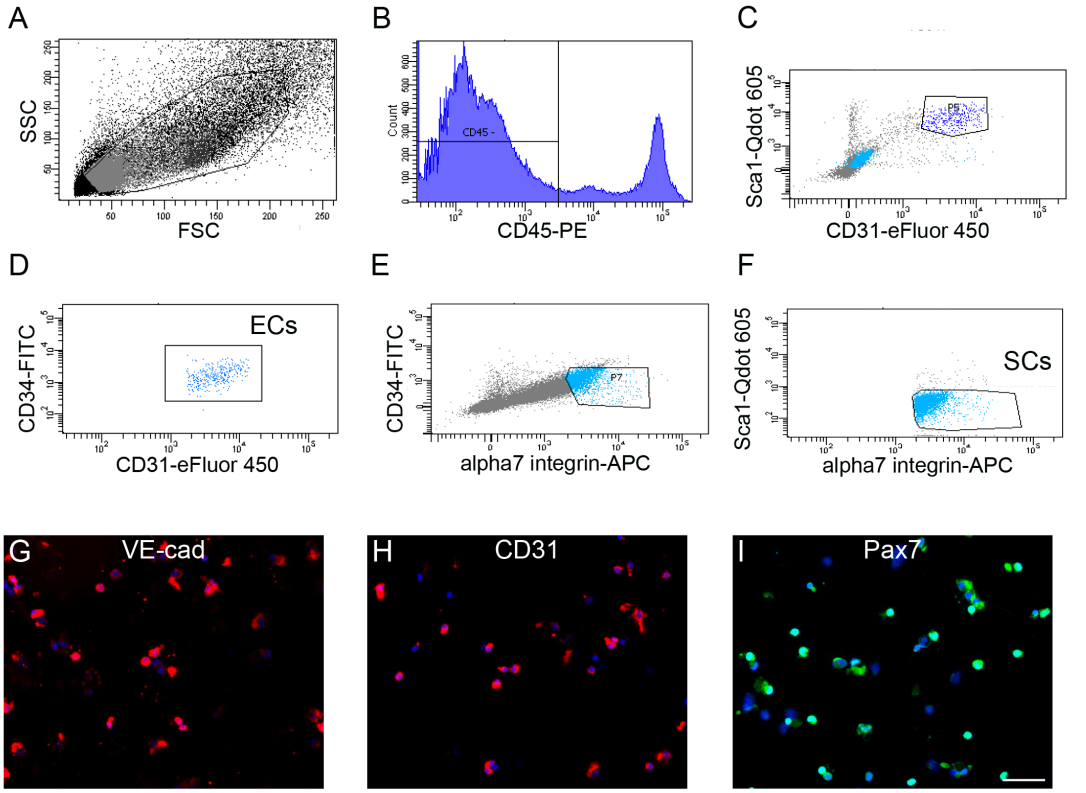

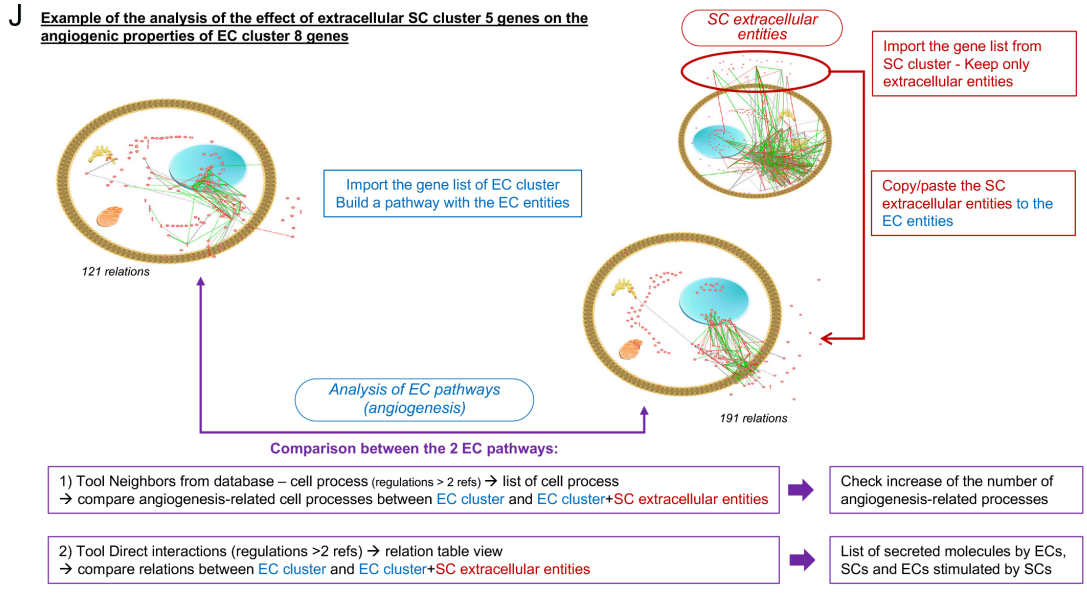

List of analyses that were performed:

Extracellular entities of:	*SC clusters 1, 5 (enriched in GO angiogenesis)*	*EC clusters 2, 4, 5, 7 (enriched in GO myogenesis)*
were tested on:	*EC clusters 2, 4, 5, 6, 7, 8 (enriched in GO angiogenesis)*	*SC clusters 3, 4, 5 (enriched in GO myogenesis)*
For properties on:	*angiogenesis*	*myogenesis*

Figure S1, related to Fig.4. Analysis of genes expressed by ECs and SCs during muscle regeneration. (A-F) Gating strategy for the FACS-sorting of ECs and SCs from regenerating *Tibialis Anterior* mouse muscle. (A) Total cell SSC/FSC representation. (B) Hematopoietic cells ($CD45^{pos}$) were excluded. (C) ECs were gated as Sca1+ CD34+ CD31+, (D) plot shows the purity of the sorted EC population. (E) SCs were isolated as CD45- CD34+ Sca1- α7-integrin+, (F) plot shows the purity of the sorted SC population. (G-I) Sorted cells were cytospined and immunostained with VE-cad (G) or CD31 (H) antibodies for ECs and Pax7 (I) antibodies for SCs (Blue=Hoechst). Bar = 50 µm. (J) Flow chart strategy for the analysis of genes expressed by ECs and SCs in regenerating muscle using Pathway Studio software. See also Figures 4, 5, 7 for the functional analysis.

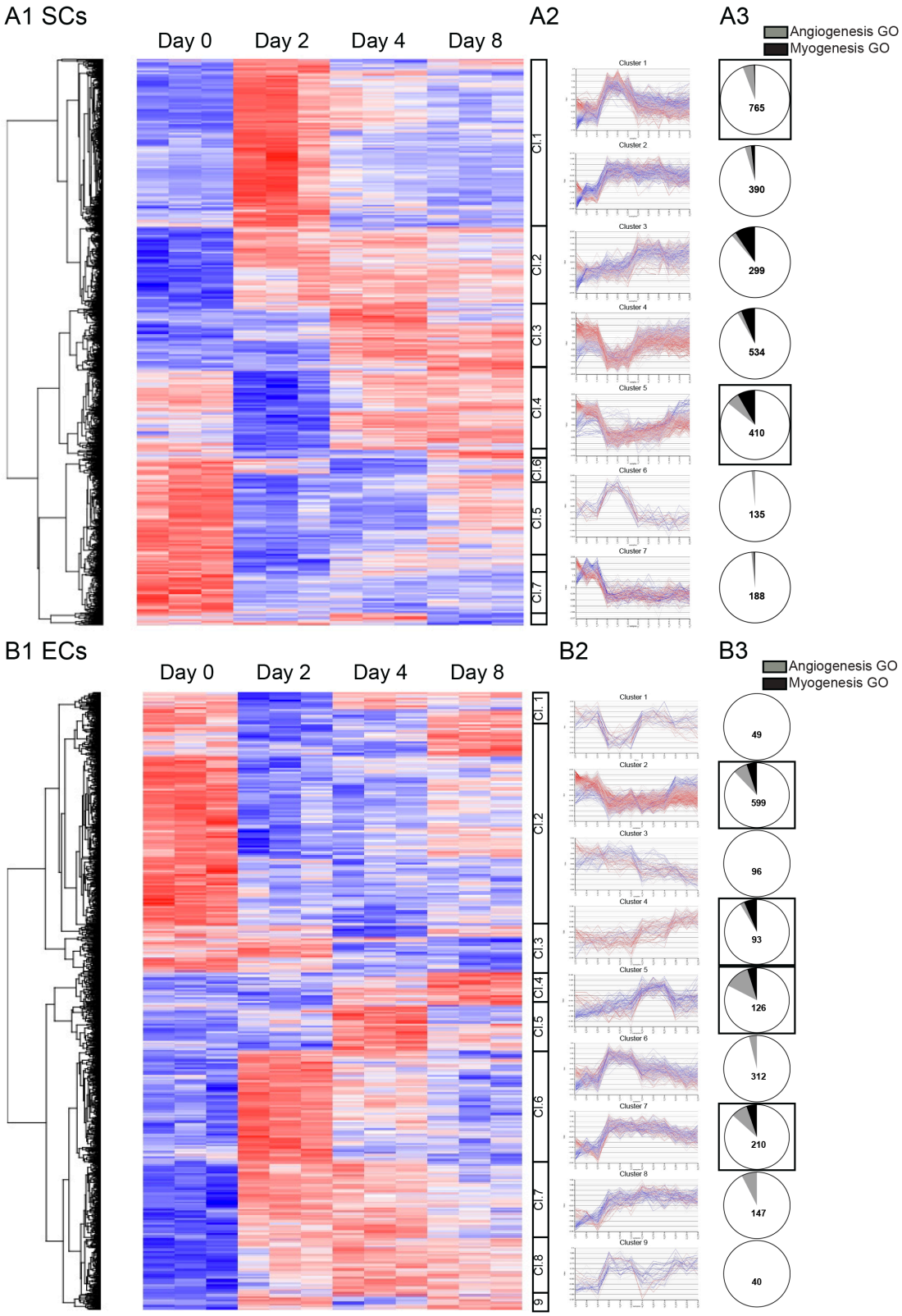

Figure S2, related to Fig.4. Molecular profiling of sorted ECs and SCs during muscle regeneration. SCs (A) and ECs (B) were isolated at different time points during skeletal muscle regeneration (days 0, 2, 4, 8). mRNAs were prepared and processed for Affymetrix-based analysis. (A1, B1) Clustering of genes expressed by SCs (A1) and ECs (A2) according to their kinetics of expression during muscle regeneration (A2, B2). (A3, B3) Gene Ontology enrichment was performed on each cluster in order to identify transcripts implicated either in angiogenesis (grey) or myogenesis (black), expressed in % of total genes in each cluster in the pie charts. Results were obtained from 3 independent experiments (see Materials and Methods section for statistical analysis). See also Figures 4, 5, 7 for the functional analysis.

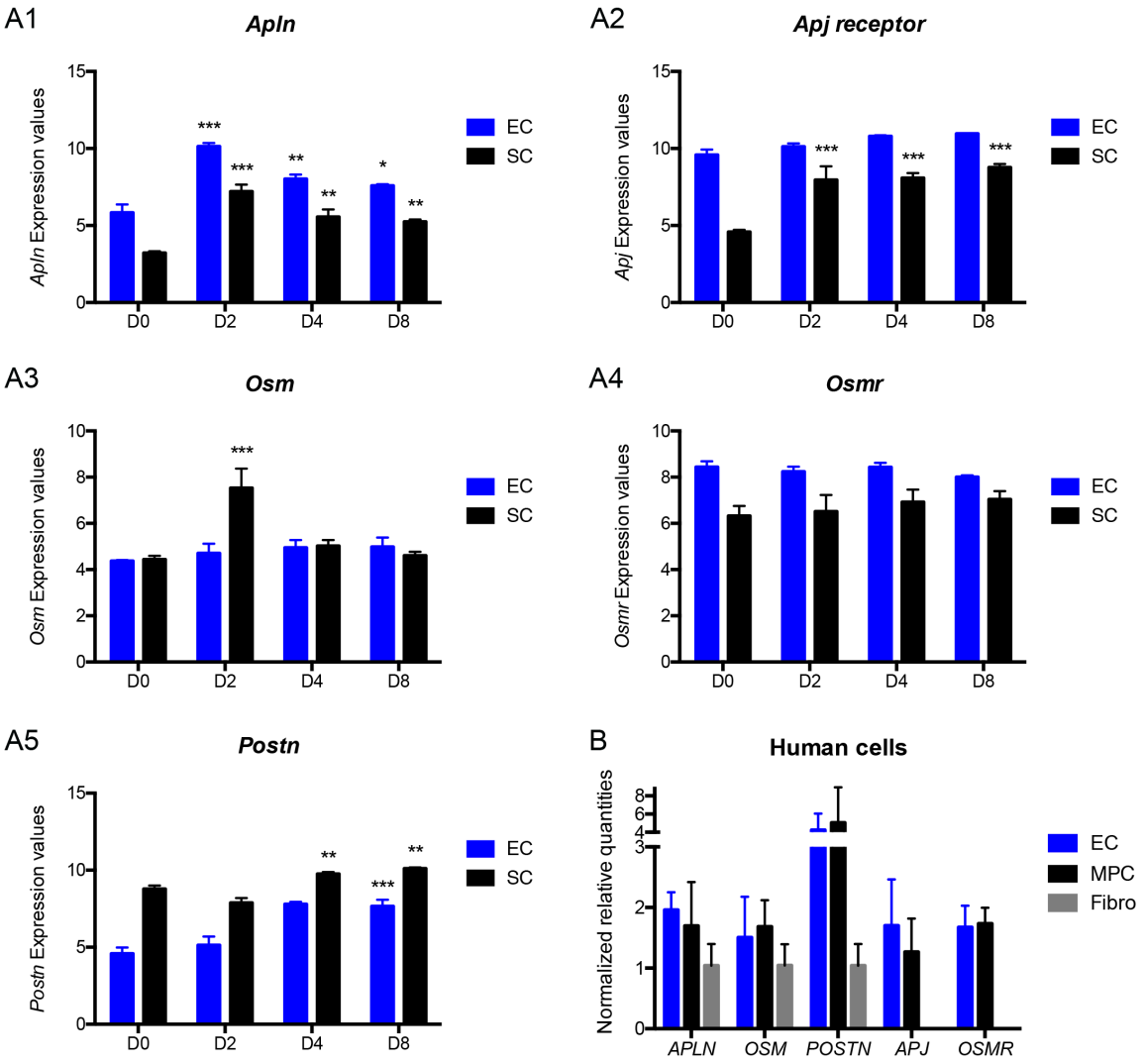

Figure S3, related to Fig.5. mRNA expression of *Apln*, *Osm* and *Postn* in murine and human cells. (A) Expression values from the transcriptomic analysis of *Apln* (A1), *Apjr* (A2), *Osm* (A3) *Osmr* (A4) and *Postn* (A5) in ECs (blue bars) and SCs (black bars) at day 0 or 2, 4 and 8 days post-injury. (B) mRNA expression of the same genes in HUVEC (blue bars), human MPCs (black bars) and Human Normal Dermal Fibroblasts (gray bars) growing *in vitro*. Results are means ± SEM of 3 independent experiments. Mann-Whitney test was performed *versus* D0 *=p<0.05; **=p<0.01; ***=p<0.001. See also Figures 4, 5, 7 for the functional analysis.

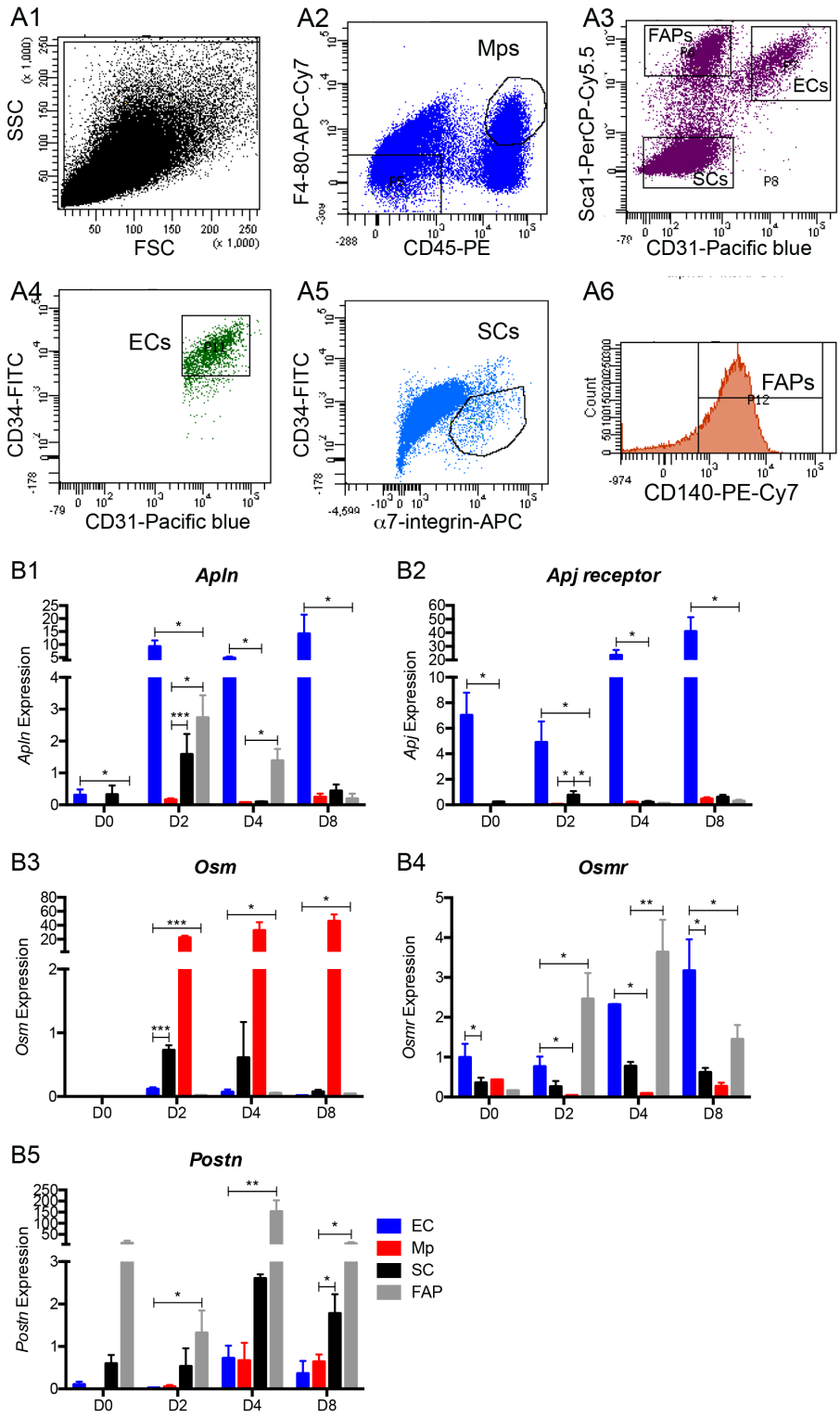

Figure S4, related to Fig.6. EC, SC, Macrophage and FAP isolation from mouse *Tibialis anterior* muscle. At Day 0 and 2, 4 and 8 days after cardiotoxin-induced damage, ECs, SCs, Macrophages (Mps) and Fibro-Adipogenic Precursor cells (FAPs) were FACS-sorted and analyzed for the expression of *Apln*, *Apjr*, *Osm*, *Osmr*, *Postn*. (A) Gating strategy: (A1) Total cell population. (A2) Mps were sorted as CD45+, F4/80+. (A3) Among CD45- F4/80- cells gated in A2, FAPs, SCs and ECs were sorted using Sca1 and CD31 labelings: (A4) Sca1+ CD31+ ECs in A3 were 100% CD34+; (A5) Sca1-, CD31- cells in A3 were further sorted as CD34lo α7 integrin+ SCs; (A6) Sca1+ CD31- FAPs in A3 were almost 100% CD140+ (PDGFRα). (B) RT-qPCR for (B1) *Apln*, (B2) *Apjr*, (B3) *Osm*, (B4) *Osmr*, (B5) *Postn* was performed on the 4 isolated cell populations. Results are means ± SEM of 3 independent experiments. Student t test was performed *versus* the other cell types *=p<0.05; **=p<0.01; ***=p<0.001 (see bars). See also Figure 6 for the functional analysis.

Table S1, related to Fig.S1, S2. List of factors secreted by ECs and SCs during skeletal muscle regeneration
See excel file

Table S2. Oligonucleotide primers used for qPCR, Related to Fig.S3, S4

Human primer	Forward sequence	Reverse sequence
CycloA	5'-GTCAACCCCACCGTGTTCTT-3'	5'-CTGCTGTCTTTGGGACCTTGT-3'
APLN	5'-ATAAGGGACCCATGCCTTTC-3'	5'-CCTCCAGAGAAGCAGACCAA-3'
APJ/APLNR	5'-ACTTCTTCATCGCCCAAACC-3'	5'-ATCCAGCACAGGGCAAAG-3'
OSM	5'-ACAGAGGACGCTGCTCAGTC-3'	5'-GGTGTCCTGCATGAGATCTGT-3'
OSMR	5'-GCAAGTCAAGGAAATGTCAGTG-3'	5'-CCCCAAGGCAGTGTCCGTCC-3'
POSTN	5'-CCATCTGTGGACAGAAAACG-3'	5'-CATGGTCAATGGGCAAAAC-3'

Murine primer	Forward sequence	Reverse sequence
Rpl13	5'-CTCTGGCCTTTTCCTTTTTG-3'	5'-CCGAAGAAGGGAGACAGTTC-3'
APLN	5'-GGAATTCGGGACCATGAATCTGAGGCTCTG-3'	5'-ACTTGGCGAGCCCTTCAATC-3'
APJ/APLNR	5'-GTGGCCACAGCAGTCTTATG-3'	5'-GAACACCATGACAGGCACAG-3'
OSM	5'-TGCTCCAACTCTTCCTCTCAG-3'	5'-CAGGTTTTGGAGGCGGATA-3'
OSMR	5'-CCAAAAAGAGTTCAGCACACC-3'	5'-CCGACCACACTTGTCTCCAT-3'
POSTN	5'-GAACCAAAAATTAAAGTCATTCAAGG-3'	5'-GGATCTTCGTCATTGCAGGT-3'

Supplemental Experimental Procedures

Human MPC Culture. Human MPCs were isolated from normal adult skeletal muscle sample according to the French legislation (protocol registered at the Agence de la Biomedecine #DC-2009-944), as previously described (Saclier et al., 2013) in HAMF12 medium (Gibco) containing 15% Fetal Bovine Serum (FBS). Conditioned medium was recovered after 24h culture in advanced RPMI 1640 medium (Gibco) containing 0.5% FBS. To obtain differentiated myocytes, MPCs were cultured for 72 h at low density (3000 cells/cm^2) in HAMF12 medium containing 5% FBS. To obtain myotubes, myocytes were seeded at high density (15000 cells/cm^2) in differentiating medium DMEM (Gibco) supplemented with Insulin and transferrin (50 mg/ml) and cultured for 72 h. In some experiments, MPCs were transduced with RFP lentivirus. The lentiviral vector particles were produced by transient transfection of the packaging construct (HIV-1 psPAX2), a minimal genome (HIV-1 SPARQ QM512-B2 from SBI) bearing the expression cassette encoding the RFP-puromycin fusion and the plasmid encoding the VSV-G-envelope expressing plasmid pMDG2 (DNA ratio 8:8:4 µg) into 293T cells (3.5 x 10^6 cells plated 1 day before transfection in 100-mm dishes) by the calcium phosphate method. Viral particles were 100X concentrated on 25% sucrose cushion and homogenized viral particles were normalized by an exogenous reverse transcriptase assay and titrated on target cellular models as in (Alais et al., 2012).

HUVEC Culture. HUVEC were obtained from Promocell and cultured in ECGM-2 complete medium (Promocell). They were transduced with GFP lentivirus and purified by cell sorting on GFP labeling (Aria III, BD Biosciences). Conditioned medium was recovered after 24h culture in advanced RPMI 1640 medium containing 0.5% FBS.

Migration assay. Migration was performed using two chambers Ibidi inserts (Ibidi GmbH). Each chamber was filled with 4500 MPCs or HUVEC for 8-12h in growth medium. Silicone walls were removed and cells were cultured in advanced RPMI medium 1640 containing 0.5% FBS. The gap between the two cell types was imaged at 0 and 24 h. The distance covered by each individual cell was measured using Image J software.

Proliferation assay. MPCs or HUVEC were seeded at 5000 cells/cm^2 in multi-well plates. HUVEC- and MPC-conditioned medium was added and cells were further cultured for 24 h. In some experiments, cells were cultured in the presence of recombinant proteins (hPOSTN 0.1, 0.5, 5, 10 µg/ml, hOSM 5, 10, 50, 100 ng/ml, APLN13 1, 10, 100, 1000 nM, Biotechne) for 24 h in HAMF12 medium containing 5% FBS. Ki67 immunolabeling or EdU staining was then performed.

Myogenic differentiation. MPCs were seeded at 5000 cells/cm^2 in multi-well plates. HUVEC-conditioned medium was added and cells were further cultured for 72h. Myogenin immunolabeling (sc-12732 Santa Cruz) was performed. In some experiments, cells were cultured in the presence of recombinant proteins (hPOSTN 0.1, 0.5, 5, 10 µg/ml, hOSM 5, 10, 50, 100 or 200 ng/ml, APLN13 1, 10, 100, 1000 nM, Biotechne) for 72 h in HAMF12 medium containing 5% FBS.

Myogenic fusion. Human MPCs were cultured for 3 days in HAMF12 medium containing 5% FBS to differentiate into myocytes. They were then seeded at 50000 cells/cm^2 in 96-well plates. HUVEC-conditioned medium was added and cells were further cultured for 72 h. In some experiments, cells were cultured in the presence of recombinant proteins (as above) for 72 h in HAMF12 medium containing 5% FBS. Desmin immunolabeling (ab32362 abcam) was performed. Fusion index was the number of nuclei in myotubes divided by the total number of nuclei.

3D *in vitro* angiogenesis assay. HUVEC-GFP were combined with the cytodex beads (Millipore) at a ratio of 400 cells/bead for 4 h at 37°C with occasional stirring. The HUVEC-coated cytodex beads were cultured overnight in a T75 flask in ECGM-2 medium, and then combined in the pre-gel solution at a concentration of 250 beads/ml. Fibrinogen type I (Sigma) was diluted to 2.5 mg/ml and supplemented with aprotinin (1/500 Sigma). MPCs, myocytes or myotubes were added to the pre-gel at various concentrations (50000 to 250000 cells/ml). In some experiments, ECs or MPCs were seeded alone and IgG, blocking antibodies or inhibitors (Biotechne) were added as follows: anti-OSM antibodies (50 µg/ml), ML221 (16 µM), anti-POSTN antibodies (40 µg/ml). Fibrin gel formation was initiated by adding 10 U/ml of thrombin (Sigma). The gels were allowed to stand for 5 min at room temperature, and then were incubated at 37°C for 15 min. In some experiments, human dermal fibroblasts (NHDF, Promocell) or MPCs or macrophages (the latter being previously activated with IL-4 or with IL-10 and Dexamethasone as described in (Saclier et al., 2013)), were seeded on top of the gel (60000 cells). The cells were cultured in EndoGro media (Millipore) up to 6 days. The medium was refreshed every 2 days. Micrographs captured the tube formation, and quantification was completed using Image J. Sprouts were measured as tubes originating from the cytodex bead. Each sprout length was calculated, as well as the presence of lumen in the sprout. For each condition, a minimum of 100 sprouts was analyzed.

Mice. Adult male C57Bl/6 mice were bred and used in compliance with French and European regulations. Principal investigators are licensed for these experiments and the protocols were approved by local Animal Care and Use Committee and the French Ministery of Agriculture.

***In vivo* muscle regeneration.** Muscle injury was caused by intramuscular injection of cardiotoxin (12µM, 50µl) in the *Tibialis Anterior* muscle, as previously described (Mounier et al., 2013). Muscles were harvested 2,4,8

days post-injury. Muscle fascia were removed, then muscles were either dissociated (for cell sorting) or snap frozen in nitrogen-chilled isopentane and kept at -80°C until use (for histological analysis). In some experiments, blocking antibodies or inhibitors (Biotechne) were injected intramuscularly as follows: blocking anti-OSM antibodies (5 µg) or ML221 (Apelin receptor APJ antagonist, 5 µg) were injected i.m. at days 2 and 3 post-injury (controls included the injection of same volume of PBS or PBS containing 2.6% DMSO). Blocking anti-POSTN antibodies (5 µg) were injected at days 3 and 4 post-injury. Muscles were collected for histological analysis at day 8.

Histology analysis. 7 µm-thick cryosections were prepared for hematoxylin-eosin staining or for immunolabeling. Muscles cryosections were treated with anti- laminin (L9393 sigma), CD31 (550274 Pharmingen), Ki67 (ab15580 abcam), Pax7 (Developmental Studies Hybridoma Bank, DSHB), Myogenin (SC-576 Santa Cruz Biotechnology), MyH3 (SC-53091 Santa Cruz Biotechnology) antibodies revealed with conjugated secondary antibodies (Jackson Immunoresearch Inc). Two-dimensional analysis was performed to evaluate the cross-section area of myofibers, the number of nuclei per fiber, the number of myogenin^{pos} cells, the number of Myh3^{pos} fibers, the number of SCs (Pax7^{pos}) per fiber, and the number of capillaries per fiber using ImageJ software. At least 300 fibers were considered for each muscle.

***In vivo* angiogenesis assay.** Primary murine MPCs were cultured in DMEM/F12 (Gibco) medium containing 20% FBS (Gibco) and 2% Ultroser G as previously described (Mounier et al., 2013). NIH3T3 cells were cultured in DMEM containing 15% FBS. Cells ($1x10^6$ cells/ml) were embedded in cold Matrigel® (BD Biosciences) and 500 µl of the mix were injected subcutaneously in 4-6-week-old male C57Bl/6 mice. In some experiments, recombinant proteins were added: mPOSTN 5 µg/ml, mOSM 50 ng/ml or APLN13 10 nM (Biotechne). After 21 days, mice were euthanized and plugs were recovered and fixed in JB fixative (zinc acetate 0.5%, zinc chloride 0.05%, and calcium acetate 0.05% in Tris buffer at pH 7.0) for 48 h and then embedded in low–melting point paraffin (Poly Ethylene Glycol Distearate, Sigma). 5 µm thick paraffin sections were deparaffinized in absolute ethanol, air dried, and used for H&E staining and immunolabeling. Sections were treated with anti- CD31 (ab28364 abcam), desmin (ab6322 abcam) and α-SMA (ab5694 abcam) antibodies and revealed with conjugated secondary antibodies (Jackson Immunoresearch Inc). Blood vessel infiltration into the plug and desmin-expressing cells (MPCs) were quantified on the totality of a plug section, by evaluating the vessel and MPC staining area using ImageJ software. Functional angiogenesis inside the plug was evaluated as the amount of hemoglobin using the Drabkin's reagent (Sigma) following the manufacturer's instructions.

Cell sorting from regenerating muscle. Single cell suspensions were obtained from muscles by enzymatic digestion (Collagenase B 10 mg/ml and Dispase 2 2.4U/ml, Roche Diagnostics). ECs, SCs, macrophages and FAPs were isolated using anti-CD45 PE, F4-80 APC-Cy7, CD34-FITC, Sca1-eFluor 605 or PerCP-Cy5.5, ✔7-integrin-APC, CD31-eFluor 450 and PDGFRα (CD140a) PE-Cy7 antibodies and their respective isotype controls (all from eBioscience except alpha7-integrin from Ablabs, British University of Columbia). Aria III (BD Biosciences) was used for cell sorting and LSRFortessa for cell analysis (BD Biosciences). All analyses and quantifications were performed using FACSDIVA software (BD Biosciences). To control cell purity, sorted MPCs and ECs were cytospined and labelled with primary antibodies against Pax7 (DSHB), VE-cad (sc-6458 Santa Cruz) and CD31 (ab28364 Abcam).

Transcriptomic analysis. Total RNAs were extracted from sorted ECs or MPCs using RNeasy Mini Kit (Qiagen). RNA integrity was checked on Agilent Bioanalyser 2100, RNA samples with >9.0 RIN value were used. Global expression data were obtained using Affymetrix GeneChip Mouse Gene 2.0 ST arrays. The microarray data are publicly available (Deposition in GEO currently in progress). Data were RMA normalized with R/Bioconductor. Data were first controlled and analyzed in an unsupervised way by PCA and a one-way ANOVA was applied to extract DEGs using PartekGS© software. Genes were selected on the global p value <0.01. A cluster analysis was then applied on selection by hierarchical clustering (Pearson for similarity and average for clustering) to find correlate genes. Enrichment analysis of each cluster was analyzed with DAVID software (https://david.ncifcrf.gov/) (Huang da et al., 2009). Pathway Studio© software (Elsevier BV) was used to identify extracellular effectors synthetized by ECs and MPCs that triggered enrichment in "myogenesis" and "angiogenesis" GOs in MPCs and ECs, respectively (strategy is presented in Figure S1J). A list of genes expressed by both ECs and MPCs was finalized (Table S1).

qRT-PCR. mRNAs of sorted or cultured cells were extracted using RNeasy Mini Kit (Qiagen). One µg of total RNA was reverse transcribed into first-strand cDNA using Superscript II Reverse Transcriptase (Life technologies). Quantitative PCR was carried out on StepOne Plus RealTime PCR system (Applied Biosystems). Reaction mixtures had a final volume of 20 µl, consisting of 1 µl of cDNA, 10 µl of Sybr Green Master (Roche) and 10 µM of primers, listed in Table S2. After initial denaturation, amplification was performed at 95°C (10 s), 60°C (5 s), 72°C (10 s) for 45 cycles. Calculation of relative expression was determined by the StepOnePlus software (Applied Biosystems) and fold change was normalized to CycloA housekeeping gene for human cells and RPL13 for murine cells.

Microscope image acquisition. Samples were observed using a Zeiss Axio Observer.Z1 microscope, and recorded at 22-24°C with a Photometrix CoolSNAP HQ2 camera using Metaview software. Magnification was

x20 for histological experiments; x20 for 2D *in vitro* experiments and x10 for 3D experiments. Mounting medium was FluoroMount-G (Interchim). Secondary antibodies were from Jackson Immunoresearch Inc and coupled with FITC, Cy3 or Cy5 fluorochromes. Nuclei were labeled with Hoechst. Pictures were analyzed using ImageJ software using manual counting.

Statistics. All experiments were performed in at least 3 independent experiments, *i.e.* different primary cultures or different animals. The exact number of experiments and statistical significance are given in the figure legends. Results are expressed as mean ± SEM. Means were compared using Mann Whitney or two-way ANOVA and Sidak post-tests.

Supplemental References

Alais, S., Soto-Rifo, R., Balter, V., Gruffat, H., Manet, E., Schaeffer, L., Darlix, J.L., Cimarelli, A., Raposo, G., Ohlmann, T.*, et al.* (2012). Functional mechanisms of the cellular prion protein (PrP(C)) associated anti-HIV-1 properties. Cell Mol Life Sci CMLS *69*, 1331-1352.

Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acid Res *37*, 1-13.