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Supplemental Information

No Identical ``Mesenchymal Stem Cells" at Different Times and Sites: Human Committed Progenitors of Distinct Origin and Differentiation Potential Are Incorporated as Adventitial Cells in Microvessels

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SUPPLEMENTAL INFORMATION

EXPERIMENTAL PROCEDURES, REFERENCES, FIGURE LEGENDS AND FIGURES

No identical "mesenchymal stem cells" at different times and sites: Human committed progenitors of distinct origin and differentiation potential are incorporated as adventitial cells in microvessels

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell isolation and culture

BMSCs were isolated and cultured as previously described (Sacchetti et al., 2007) from surgical waste from long bones, or iliac crest bone marrow aspirates. PE cells were generated as per established methods (Cicconetti et al., 2007). Human CB cells (≥36wks of gestation) were isolated and cultured as described previously (Kluth et al., 2010). Purchased human dermal fibroblasts (PromoCell GmbH, Heidelberg, Germany) were cultured in DMEM-high glucose (Invitrogen), supplemented with 2mM glutamine. Human amniotic cells were isolated as previsouly described (Pievani et al., 2014). HUVECs were grown in Clonetics EGM-2 BulletKit (Cambrex Corporation) following the manufacter's instructions.

Muscle-derived cells were isolated from normal skeletal muscle (1-30x10^2mg) from 17 human adult patients (aged 25-65 yrs) undergoing orthopedic surgery [*vastus lateralis* (1), *quadriceps femoris* (5), *triceps brachii* (2), *deltoides* (4), *gluteus maximus* (5)]. Samples were washed with Hank's balanced salt solution without Ca^{2+}/Mg^{2+} (HBSS, Invitrogen Life Technologies Corp) containing 30mM HEPES (Sigma), 100U/ml penicillin, 100μg/ml streptomycin (Invitrogen) for 10min at room temperature with gentle agitation. Tissue samples were used to obtain single cell suspensions by digesting twice with 100U/ml *Clostridium histolyticum* type II collagenase (Invitrogen) supplemented with 3mM $CaCl_2$ in

Ca^{2+}/Mg^{2+}-free PBS (Invitrogen) for 40 min at 37°C with gentle agitation. The samples were centrifuged at 1000 rpm for 5min at 4°C, washed with Ca^{2+}/Mg^{2+}-free PBS, resuspended in PBS, passed through 18 gauge needles to break up cell aggregates, and filtered through a 40μm pore size cell strainer (Becton Dickinson) to obtain a single cell suspension. Nucleated cells were counted using a haemocytometer.

Human adipose tissue-derived cells were obtained from human adult subcutaneous adipose tissue. Fat tissue was minced with scissors, washed with Ca^{2+}/Mg^{2+}-free PBS and the extracellular matrix was digested with collagenase type I (Invitrogen), at 37°C for 1h. The samples were centrifuged at 1000 rpm for 5min at 4°C, washed with Ca^{2+}/Mg^{2+}-free PBS, resuspended in PBS, passed through 18 gauge needles to break up cell aggregates, and filtered through a 40μm pore size cell strainer to obtain a single cell suspension.

RT-PCR Analysis

From CD146-sorted, uncultured cells, total RNA was extracted using a PicoPure™ RNA Isolation Kit (Arcturus Bioscience), per the manufacturer's instruction. cDNA was synthesized using 9μl of RNA, 100ng of random hexamers, and 50u of SuperScriptII Reverse Transcriptase (Invitrogen) in a total volume of 20μl. From cultured cells, total RNA was extracted using TRIZOL™ RNA isolation system (Invitrogen) per the manufacturer's instructions. cDNA was synthesized using 3μg of RNA, 150ng of random hexamers, and 50u of SuperScript II Reverse Transcriptase (Invitrogen) in a total volume of 20μl. Target cDNA sequences were amplified in standard PCR reactions using Platinum® PCR SuperMix according to the manufacturer's instructions. Primers used for RT-PCR are described in Supplemental Table 7C.

Gene expression profiling and data analysis.

Total RNA was isolated from multi-clonal cultures of $CD146^+$ cell populations after 2wks of culture in basal culture conditions (αMEM (Invitrogen) with 20% FBS (Invitrogen), 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin) using RNeasy RNA isolation kit (Qiagen) per the manufacturer's instructions. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/integrity of RNA samples using an Agilent 2100 bioanalyzer. cDNA synthesis, biotin-labeled target synthesis, HG-U133 plus 2.0 GeneChip (Affymetrix) array hybridization, staining and scanning were performed according to the standard protocol supplied by Affymetrix. Probe level data were normalized and converted to expression values using Partek Genomics Suite 6.2 (Partek Inc), following the RMA algorithm (Irizarry RA, et al. 2003) or DChip procedure (invariant set) (Li and Wong, 2001; Li and Wong, 2001). Quality control assessment was performed using different Bioconductor packages such as R-AffyQC Report, R-Affy-PLM, R-RNA Degradation Plot and Partek's QC. Low quality samples were removed from analysis. Before significance analysis, Partek's batch correction method, which reduces variation due to random factors, was used to enhance signal. Sample data were then filtered in order to remove probesets having a standard deviation/mean ratio greater the 0.8 and less that 1000. Principal Component Analysis (PCA) as well as the unsupervised hierarchical clustering were performed using Partek GS®. The agglomerative hierarchical clustering was performed using the Euclidean distance and the average linkage method. Differentially expressed genes were selected using a supervised approach using the ANOVA package included in Partek GS® Software. Formally, an unpaired t-test using a contrast fold change of at least 3 and an FDR (q-value) <0.05 was used in order to perform multiple pairwise comparisons between each class and the rest. Raw data of gene expression profiling were submitted to GEO repository (GSE69991).

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed using GSEA software [(http://www.broadinstitute.org/gsea/index.jsp; (Subramanian et al., 2005)] on log2 expression data of $CD146^+$ cell population purified from BM, CB, MU and PE and classified in the corresponding classes. Gene sets were taken from the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). In particular, we investigated whether each class of $CD146^+$ cells was associated with over- or under-represented genes in pairwise comparisons between each class and the rest. Gene sets significantly over- or under-represented were returned by GSEA as showing an Enrichment Score ES<0 and an FDR<25% when using Signal2Noise as metric and 1,000 permutations of phenotype labels.

Secondary Passage of $CD146^+$ MU CFU-Fs (Self-renewal)

Muscle $CD146^+/CD34^-$ cells were injected i.m. into the left *tibialis anterior* as described in the text. At 6wks, animals were euthanized and the injected and contralateral (control) *tibialis anterior* were harvested, washed with Ca^{2+}/Mg^{2+}-free PBS and the tissue was digested with collagenase type II (Invitrogen) at 37°C for 1h. The samples were centrifuged at 1100rpm for 5min at 4°C, washed with Ca^{2+}/Mg^{2+}-free PBS, resuspended in PBS, passed through 18 gauge needles to break up cell aggregates, and filtered through a 40μm pore size cell strainer to obtain a single cell suspension. ~$5x10^5$ cells were obtained from the digestions. For flow cytometry, dissociated cells were incubated with mouse monoclonal antibodies specific for human CD146, human CD44 and human CD90. The isolated human cells were plated in culture and analyzed by FACS at 2wks for expression of hCD146, hCD90, and hCD44. Human cells were magnetically separated based on CD44 and CD90 expression using MiniMacs (Miltenyi). To assay for secondary MU-CFU-Fs, positive cells were recovered and resuspended in medium; cells were plated in culture at clonal density (1.6 cells/cm^2) and scored for colony formation at

2wks. The discrete colonies obtained were harvested and analyzed by FACS for expression of hCD56, hCD146 and hCD34.

Immunohistochemistry studies

Orthotopic and heterotopic transplants were snap-frozen in OCT embedding medium in liquid nitrogen and cryostat-sectioned serially, or alternatively fixed in 4% formaldehyde (and decalcified in the case of bone- or ceramic-containing transplants) and processed for paraffin embedding. Five-μm thick paraffin sections were stained with H&E, Safranin O and Light Green, Alcian blue or Sirius red for histology. All primary antibodies used for immunolocalization studies are listed in Supplemental Table 13 and were used as per standard immunoperoxidase (DAB reaction) or immunofluorescence protocols. Secondary antibodies labeled with Alexa Fluor 594 and 488, were from Molecular Probes (Invitrogen). Nuclei were stained by DAPI or Propidium Iodide (Sigma). Fluorescence images-stacks were obtained using confocal microscopy laser scanning (Leica TCS SP5, Leica Microsystems). Brightfield light and polarized light microscopy images were obtained using Zeiss Axiophot microscope (Carl Zeiss)**.**

Microvessel density analysis

Microvessel density analysis was performed as described (Melero-Martin J.M. et al., 2008). Microvessels were quantified by evaluation of 10 randomly selected fields of H&E stained sections taken from the transplants. Microvessels were identified as lumenal structures containing red blood cells and counted. Microvessels density was reported as the average number of red blood cell filled microvessels from the fields analyzed and expressed as vessels/mm^2. Values reported for each experimental condition correspond to the average values ± S.D. obtained from at least three individual mice.

Knockdown of CD146 in BM progenitors

Short hairpin (sh) sequences (19nt) targeted to human CD146 exon 6, 8 and 15 were designed using algorithms in the public domain (http://www.ambion.com/techlib/misc/siRNA_finder.html), submitted to BLAST analysis to exclude off-target annealing, and custom-synthesized (Operon Biotechnologies GmbH, Cologne, Germany). The control 19nt sequence was designed to not match any sequence in the human genome. The shRNA duplexes were cloned into ClaI/MluI sites of the pLVTHM-eGFP lentiviral transfer vector (from D. Trono, Ecole Politechnique, Genève Switzerland; maps at http://www.tronolab.com), downstream of the H1 promoter. Lenti-viral vectors were produced as described (Piersanti et al., 2006), by transfecting 293T cells with the transfer vector, the packaging vector pCMV-dR8.74 and the VSV-G envelope vector pMD2G (http://www.tronolab.com). BMSCs were infected with each lentivirus as described (Piersanti et al., 2006). Efficiency and efficacy were assessed by western blot analysis and FACS (CD146). The lenti-viral vectors encoding shRNA targeted to CD146 exon 15 (LV-shCD146) was chosen as the most effective and used or experiments at an MOI of 1.

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SUPPLEMENTAL FIGURES

Supplemental Figure 1. Transcriptome, RT-PCR and fluorescent immunocytochemistry analyses of myogenic markers in "MSCs." A) Analysis of the transcriptome of BM (bone marrow), CB (cord blood), MU (muscle) and PE (periosteum) cells shows signification expression of PAX7 only in MU cells (circles – replicates, boxes – average $\pm$ SD). Results are derived from 3 independent cultures of each cell type. B) RT-PCR analysis of myogenic regulators and markers in "MSCs," demonstrating restriction of their expression to $CD146^+$ muscle-derived cells only (sorted a and b – two independent populations of cells). C) Sorted and cultured cultures $CD146^+/CD56^-$ muscle-derived cells progressively turn on expression of human-specific myogenic markers PAX3, Myf5 and Desmin. Results for (B) and (C) are representative results from 1 of at least 3 independent experiments.

Supplemental Figure 2. Transcriptome analysis for hematopoietic cytokines, and in vivo transplantation of "MSCs." A) Expression of hematopoiesis-supportive factors by BM (bone marrow)-, CB (cord blood)-, MU (muscle)- and PE (periosteum)-derived cells. Results are derived from 3 independent cultures of each cell type. Hematopoietic factors are highly over expressed in BM cells compared with the others.

In vivo differentiation of bone marrow-(B), dermis-(C), adipose-(D) and amniotic fluid-derived cells (E). When cultured "MSC" cell strains were transplanted, using the same *in vivo* assays and HA/TCP as a carrier and stained with Sirius red, osteogenic potential was restricted to bone marrow-derived cells (BM). Cells derived in culture from dermis (D), adipose tissue (A) or amniotic fluid (AF), regularly failed to form any histological bone, whereas "MSCs" from BM did form bone and establish the hematopoietic microenvironment *in vivo* (* in panel B) (Bars=300μm).

Supplemental Figure 3. Self-renewal of MU $CD146^+$ cells. Muscle $CD146^+/CD34^-$ cells were injected i.m. into the left *tibialis anterior*. At 6 wks, animals were euthanized and the injected and contralateral (control) *tibialis anterior* were harvested. Subsequently, collagenase-released cells of the harvested injected TA were used to perform secondary MU colony forming efficiency assays. Human cells were isolated based on hCD44, hCD90 and hCD146 expression and after brief expansion (2 wks), reanalyzed by FACS for hCD44, hCD90 and hCD146 expression. The human cells were the isolated by MiniMacs (Miltenyi), and were replated in culture at clonal density. All colonies harvested at 2 wks were uniformly positive for hCD146 and negative for hCD56 (a mature myogenic marker) and hCD34 (a hematopoietic/endothelial marker), demonstrating that $hCD146^+$ pericytes isolated from the injected muscle were the source of the secondary MU-CFU-Fs, indicative of self-renewal. Results are representative of 1 out of 2 independent experiments.

Supplemental Figure 4. In MU cells, expression of CD146, CD56 and CD34 by FACS fluorescent, immunohistochemistry and immunocytochemistry, and colony forming efficiency. A) Isotype control and dual label FACS analysis of a collagenase-released muscle cell suspension. Expression of CD146 and CD56 is mutually exclusive in distinct cell subsets, with no co-expression. B) Localization of CD146 and CD56 in muscle sections. CD56 is restricted to the surface of myofibers where satellite cells reside

(*arrows* in b, *red arrows* in e). CD146 is restricted to vascular walls (*arrows* in d, *green arrows* in e). MF, myofiber. Bars=70μm in a-d, 20μm in e. C) CFE assay with $CD146^{\pm}/CD56^{\pm}$ subsets of collagenase-released cells. CFU-Fs capable of growth on plastic are found in the $CD146^{+}/CD56^{-}$ fraction. Numbers (upper left each panel) indicate the number of cells plated/cm^2. D) Isotype control and dual label FACS of CD146 and CD34 expression in collagenase-released muscle cells. ~25% of CD146+ cells co-express CD34 (ellipse), and ~12% of CD34+ cells express CD146. E) Localization of CD146 and CD34 in muscle sections. a) Both antigens are localized to cross-sections of pre-capillary arterioles and post-capillary venules (a, *large arrows*), whereas most capillaries (a,i,j, *small arrows*) among myofibers (MF) only label for CD34. b-d) Detail of a large pre-capillary arteriole. Endothelial cells [*e* in d] co-express CD34 and CD146; subendothelial mural cells [*peri* in d] only express CD146. e-g) Detail of a small pre-capillary arteriole. Endothelial cells express CD34 but not CD146. Subendothelial cells express CD146 but not CD34. h-j) Detail of a capillary adjacent to a myofiber (MF). Endothelial cells express CD34 but not CD146. No CD146 expression is detected. Bar=90μm in a; 50μm in b-g; 10μm h-j. F) When sorted $CD146^{+}/CD34^{-}$, $CD146^{+}/CD34^{+}$ and $CD146^{-}/CD34^{+}$ total collagenase-released cells plated at clonal density are induced to myogenic differentiation (2% HS on Matrigel™), high numbers of myotubes expressing skeletal muscle-specific myosin heavy chain (MyHC) are found in freshly sorted cultures enriched in muscle-derived $CD146^{+}/CD34^{-}$ cells. Only rare myotubes are observed in $CD146^{+}/CD34^{+}$ (Bars=100μm or 120μm).

Supplemental Figure 5. Formation of vascular networks by "MSCs" *in vivo*. MU (a,d), CB (b,e) and BM (c,f) cells with HUVECs (a-c) or alone (d-f) were resuspended in Matrigel and implanted on the backs of SCID/beige mice by subcutaneous injection. Implants were harvested after 21 days and stained with H&E. H&E staining revealed the presence of lumenal structures containing erythrocytes (black arrow heads) in implants where both cells types (HUVECs and MU, CB or BM cells) were used (a,b,c)

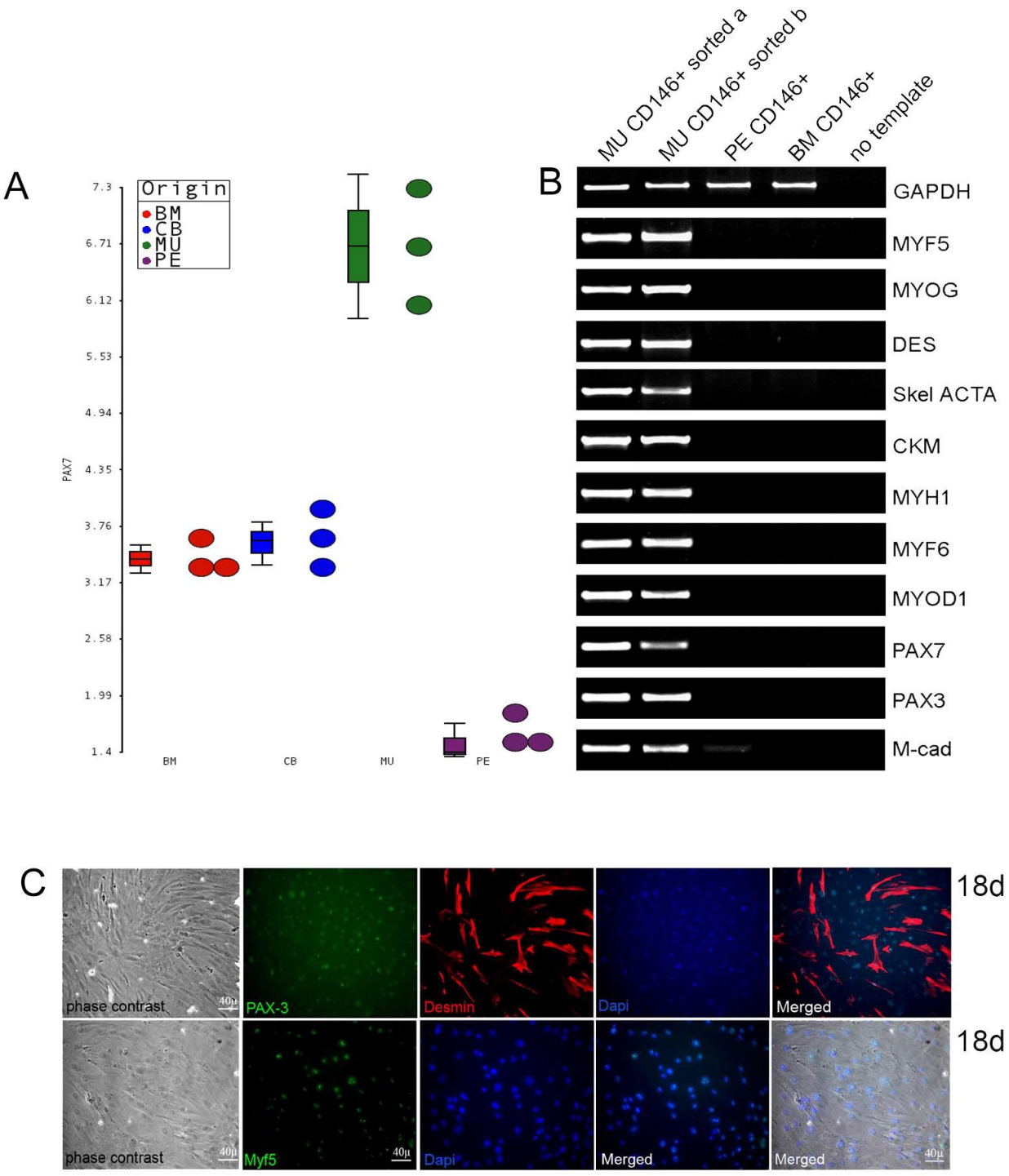
but not in implants where MU (d), CB (e), or BM (f) were used alone (Bars=100 μm). Images are representative of implants harvested from at least 3 different mice. Quantification of microvessel density was performed by counting erythrocyte-filled vessels in implants (g; n=3 each condition). Each bar represents the mean±SD (vessels/□m^2) obtained from the vascularized implants, *$p<0.01$. Results are from 1 experiment representative of at least 3 independent experiments.

Supplemental Figure 6. Effect of knockdown of CD146 in BMSCs in vessel formation with HUVECs in an *in vivo* transplantation assay. A) Co-transplant at 3 wks of BM-derived progenitors (green, cell-surface GFP) transduced with a control lentiviral shRNA (LVsh-Ctr) in Matrigel™, along with HUVECs (red, hCD34) resulting in the formation of a well organized capillary lattice. One can clearly see that the structures formed have a lumen (*) created by endothelial cells that are surrounded by BM cells (arrows). B) Co-transplant at 3 weeks of in BM-derived progenitors in which CD146 was knockdowned by shRNA (LVshCD146) in Matrigel™, along with HUVECs. In this case, structures that are formed are of irregular size and shape (arrows, compare with panel A). In many instances, they were devoid of a lumen (**), and devoid of a regular mural cell coat (arrows), or completely disorganized (arrow heads). In many instances, there is no association between the endothelials cells and the BM cells at all (Bars=20μm, 80μm, 90μm, 130μm or 150μm). Results are from 1 experiment representative of at least 3 independent experiments.

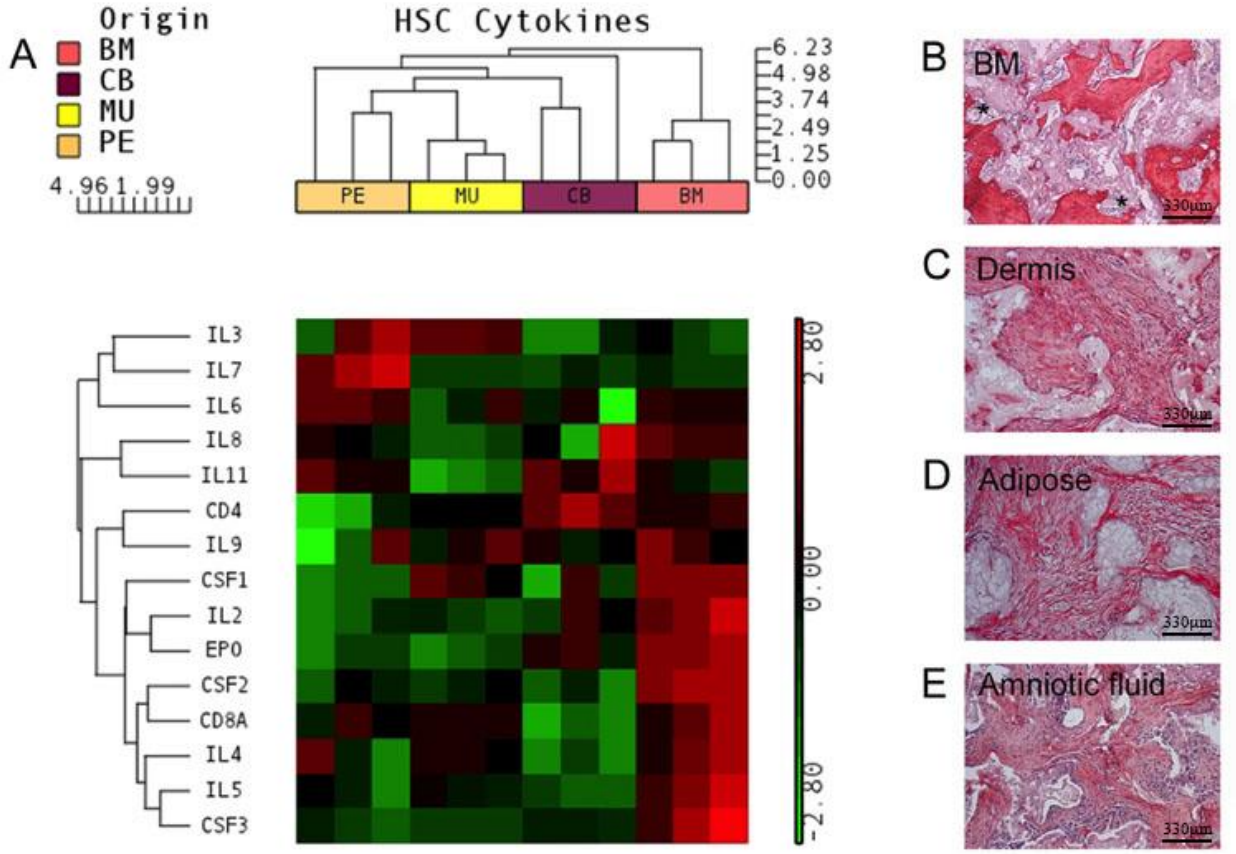
Supplemental Figure 7. Comparison of pericyte-related genes expressed by "MSCs." BM (bone marrow), MU (muscle), CB (cord blood) and PE (periosteum) cells expressed several pericyte-related genes: however, no cell type expressed all of them, and the pattern of expression varied by cell type,

consistent with their diverse developmental origins. Results were derived from the analysis of 3 indpendent cultures for each cell type.

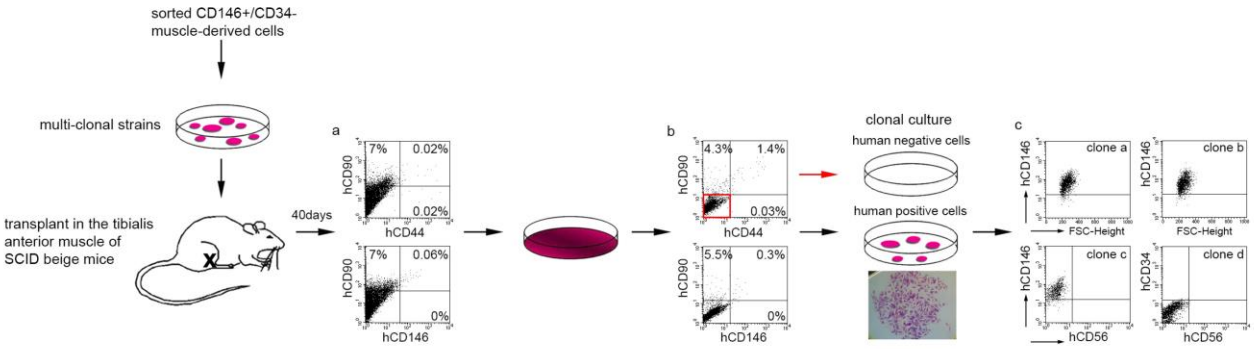
Supplemental Figure 1. Transcriptome, RT-PCR and fluorescent immunocytochemistry analyses of myogenic markers in "MSCs."



Supplemental Figure 2. Transcriptome analysis for hematopoietic cytokines and in vivo transplantation of "MSCs."



Supplemental Figure 3. Self-renewal of MU CD146^{+} cells.



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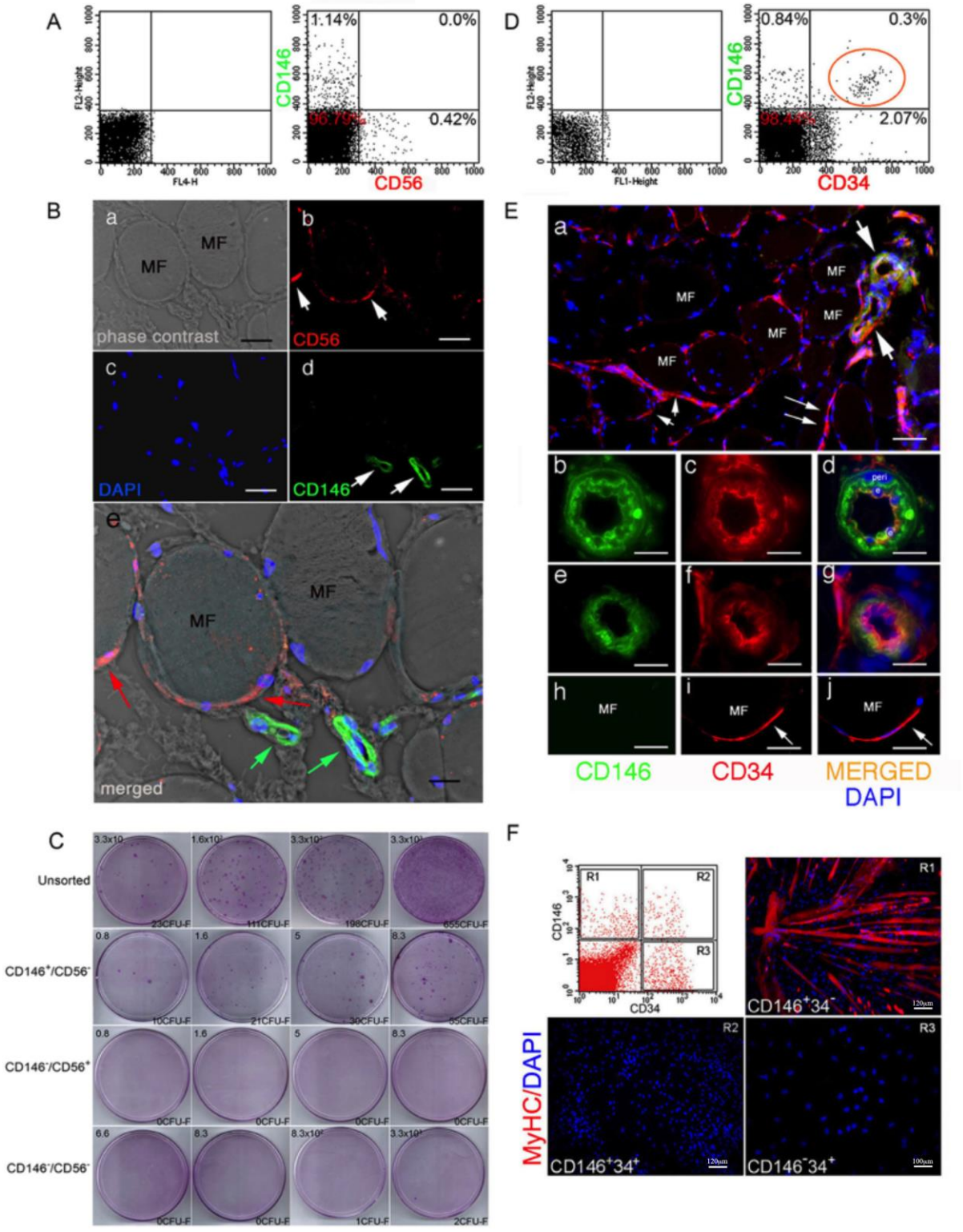
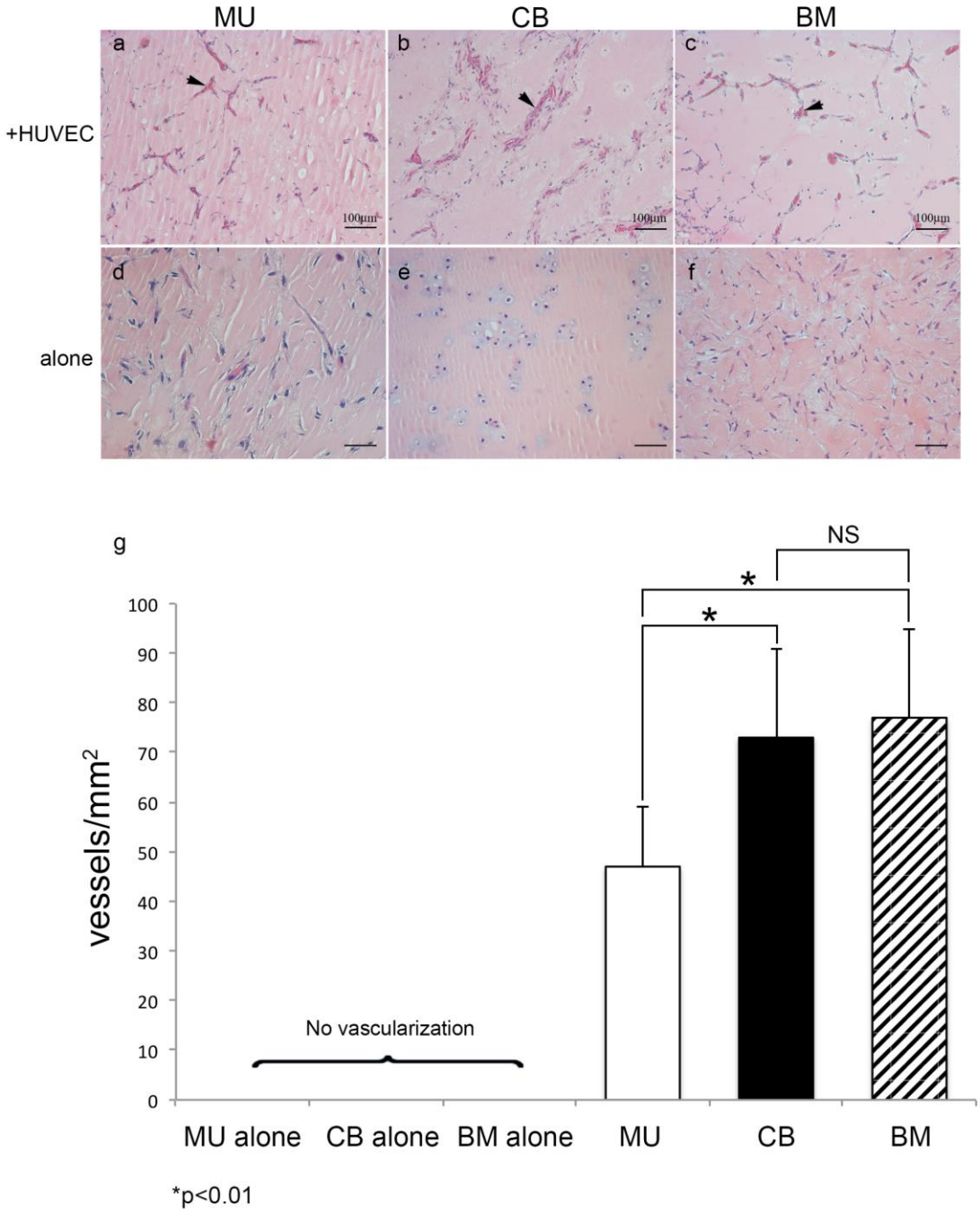


Supplemental Figure 5. Formation of vascular networks by "MSCs" *in vivo*.



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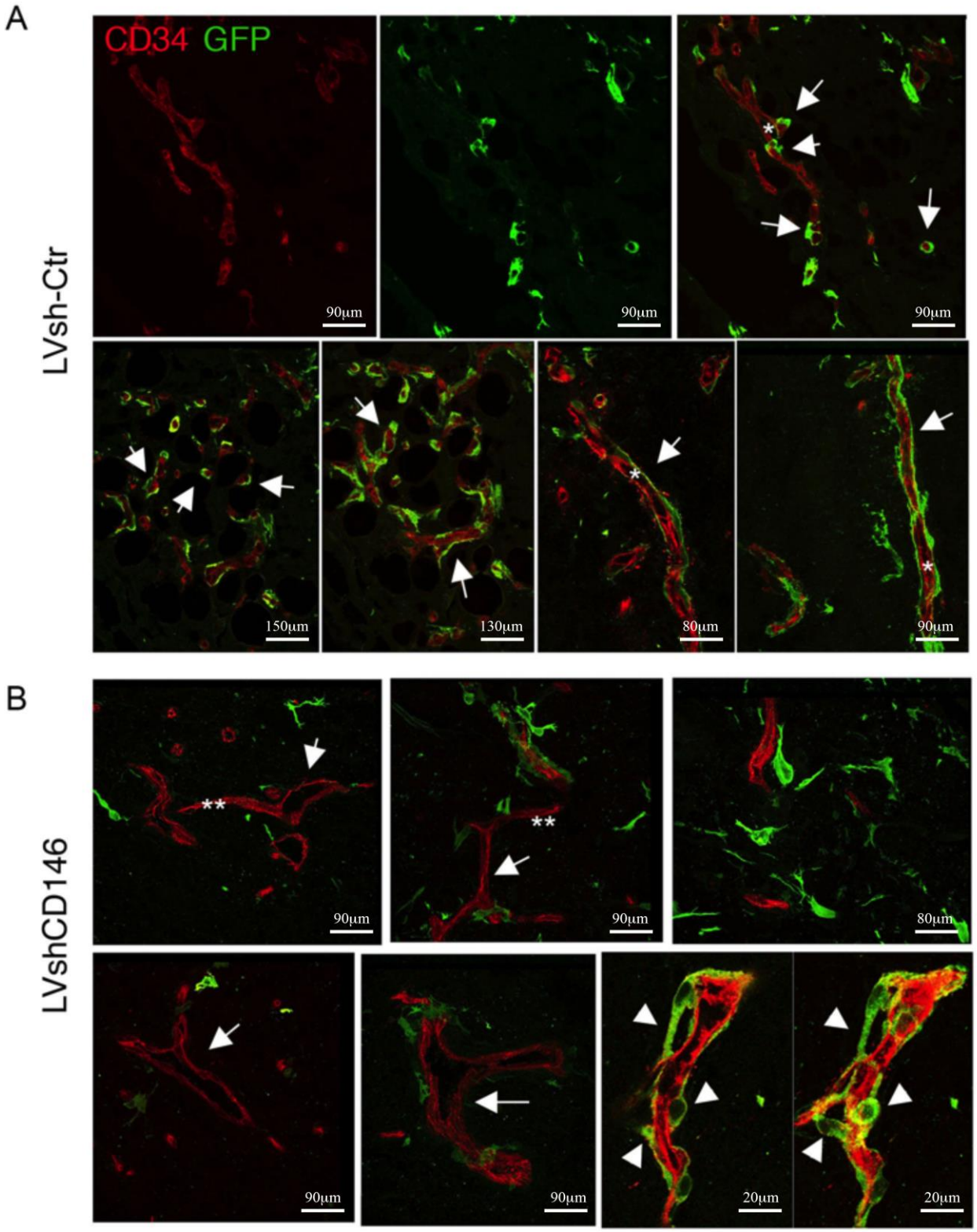
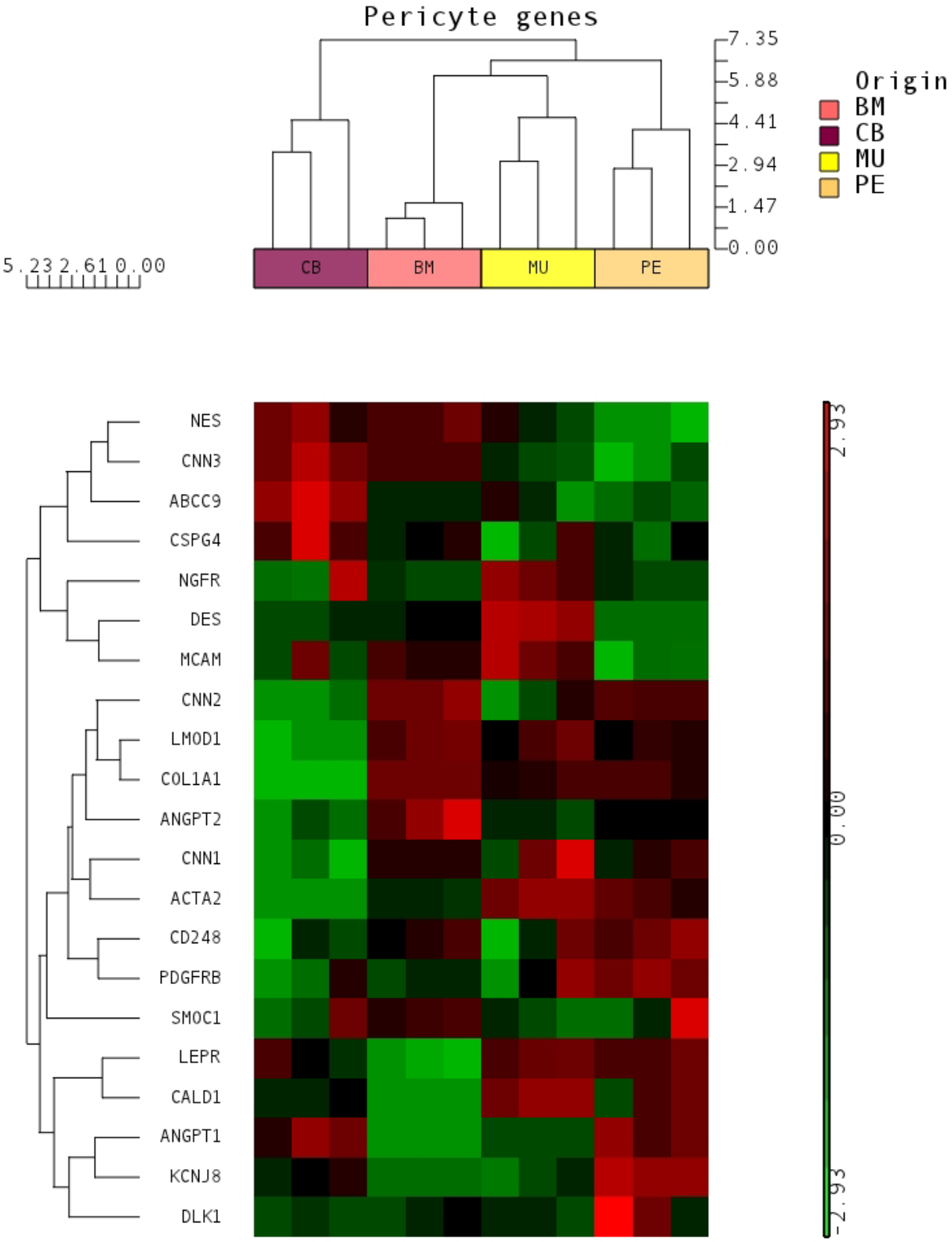


Supplemental Figure 7. Comparison of pericyte-related genes expressed by "MSCs."



Supplemental Table 5. Quantification of satellite, mural cells and regenerative efficiency of CD146$^+$/CD34$^-$ cells *in vivo*

A. Quantification of satellite, mural* cells *of CD146$^+$/CD34$^-$ cells in SCID beige/ctx

Donor cell types huCD146$^+$/CD34$^-$	*Strain of mice*	*delivery*	*Number of injected cells*	*days after injection*	*% positive cells (mean±dev st)*
huCD56 (N-CAM)	SCID beige	i.m.	$1x10^6$	40	8.8 ± 2
huCD146 (M-CAM)	SCID beige	i.m	$1x10^6$	40	0.03 ± 0.01
huLamin A/C	SCID beige	i.m.	$1x10^6$	40	9.2 ± 2.7

B. Quantification of satellite, mural cells of CD146$^+$/CD34$^-$ cells in SCID/mdx

Donor cell types huCD146$^+$/CD34$^-$	*Strain of mice*	*delivery*	*Number of injected cells*	*days after injection*	*% positive cells (mean±dev st)*
huCD56 (N-CAM)	SCID/mdx	i.a.	$2x(5x10^5)$	15	2.6 ± 1.8
huLamin A/C	SCID/mdx	i.a.	$2x(5x10^5)$	15	3 ± 1.4

C. Regenerative efficiency of CD146$^{+/}$CD34$^-$ cells in vivo

Donor cell types huCD146$^+$/CD34$^-$	*Strain of mice*	*delivery*	*Number of injected cells*	*days after injection*	*N° positive fibers per muscle (mean±dev st)*
huSpectrin	SCID beige	i.m.	$1x10^6$	40	406 ± 47
huDystrophin 2	SCID/mdx	i.a.	$2x(5x10^5)$	15	543 ± 51.8
huDystrophin 3	SCID beige	i.m	$1x10^6$	40	503 ± 28.5

i.m., single intra-muscolar injection into tibialis anterior; ctx, cardiotoxin.
i.a., two consecutive intra-femoral artery injections.
i.m., single intra-muscular injection into *tibialis anterior*; i.a., two consecutive intra-femoral artery injections.
In all injected animals, only the *tibialis anterior* was analysed.

Data are the average of at least two independent experiments.

Supplemental Table 6. CFE assays for unsorted and sorted muscle cells.

A. CFE assay for unsorted, CD146^{+} and CD146^{-} muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*		
		Unsorted	CD146^{+}	CD146^{-}
1x10^2	1.6	2 ± 1.7	24 ± 21	0
2x10^2	3.3	ND	82 ± 2	0
1x10^3	16	10.3 ± 0.6	confluent	0
1x10^4	160	90 ± 20.5	confluent	0
1x10^5	1600	293 ± 47.4	ND	0

B. CFE assay for sorted CD146$^{+/-}$ and CD56$^{+/-}$ muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*		
		CD146^{+}/56^{-}	CD146^{-}/56^{+}	CD146^{-}/56^{-}
1x10^2	1.6	41.7 ± 4	0	ND
2x10^2	3.3	83.5 ± 8	0	ND
3x10^2	5	117.3 ± 0.4	0	ND
5x10^2	8.3	195.5 ± 0.7	0	0
1x10^3	16	ND	ND	0
1x10^4	160	ND	ND	0
1x10^5	1600	ND	ND	1 ± 1

C. CFE assay for sorted CD146$^{+/-}$ and CD34$^{+/-}$ muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*			
		CD146^{+}/34^{-}	CD146^{+}/34^{+}	CD146^{-}/34^{-}	CD146^{-}/34^{+}
1x10^2	1.6	16.5 ± 6.4	4 ± 1.5	0	0
3x10^2	5	31.5 ± 2.1	12 ± 4.2	0	0
5x10^2	8.3	52.5 ± 3.5	14 ± 1.4	0	0
1x10^3	16	ND	ND	0	0
1x10^4	160	ND	ND	0	0
1x10^5	1600	ND	ND	0	1 ± 1

D. CFE assay for sorted CD146^{+}/ALP$^{+/-}$ and CD146^{-}/ALP^{+} muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*	
		CD146^{+}/ALP$^{+/-}$	CD146^{-}/ALP^{+}
5	0.083	2 ± 0.7	0
50	0.83	21 ± 0.7	0
1x10^2	1.6	39.8 ± 0.4	0
2x10^2	3.3	79.5 ± 0.7	0
3x10^2	5	ND	8.2 ± 8.8
1x10^3	16	ND	28 ± 28

CFU-F, Colony Forming Unit-Fibroblastic; ND, not determined. Data are expressed as mean ± SEM. Data are from 2 independent experiments, each one done in triplicate.