Supplementary Materials and Methods

Quantitative reverse-transcription real-time PCR (qRT-PCR)

Primer pairs used for generating the data shown in the supplementary figures are given in Supplementary Table S1.

Flow cytometry and immunohistochemistry

The antibodies used in immunofluorescence staining were anti-LYVE1 (eBioscience), -CD105 (Biolegend), -vWF (Dako) and -ZO1 (Chemicon).

Cell culture

For VEGF inhibition, 1 ng/ml of VEGFR2-Fc (R&D Systems, Inc.) was added to the medium once every three days. For the transdifferentiation assay, TGFβ1 or TGFβ2 (R&D Systems, Inc.) was added to the medium at day 8 of culture and the appearance of SMA^+ cells was evaluated 48 hours later. For the hematopoietic cell differentiation assay, MethoCult GF M3434 (Stemcell Technologies) was used according to the manufacturer´s instructions. Briefly, $1x10^3$ KSL hematopoietic stem cells or $1x10^3$ EC-SP cells were mixed in the medium and cultured in 24-well plates.

For tumor EC-SP analysis, subcutaneous xenografts were established by injecting 3×10^6 Lewis Lung carcinoma cells into the flanks of mice. Tumor size was measured with calipers. Tumor volume was calculated according to the formula V=1/2×length×width×height.

BrdU retention assay

BrdU (100 mg/kg, Sigma) was injected into mice daily at P3, P4 and P5 and maintained for 7 weeks. EC-SP and EC-MP cells were sorted and cytospun onto slides. TO-PRO3 (Invitrogen) and anti-BrdU (Abcam) antibody were used to enumerate BrdU-retaining cells.

CFU-S assay and BM reconstitution analysis

8 week-old C57BL/6 male mice were used as recipients for transplantation of BM cells or EC-SP cells from EGFP mice. Recipients were sublethally irradiated with 4.0 Gy before transplantation and intravenous infusion of $3x10^3$ KSL cells or EC-SP cells. For CFU-S assays, mice were analyzed after 12 days and for BM reconstitution, after 2, 4 and 8 weeks.

Supplementary Figure legends

Supplementary Figure S1. EC-SP cells do not express hematopoietic markers.

Histogram showing expression of surface markers on EC-SP cells (green) and $CD45^+$ cells (red) in the digested muscle. EC-SP cells were negative for all the hematopoietic markers.

Supplementary Figure S2. EC-SP cells are not fibroblasts, pericytes/mural cells or hematopoietic cells.

(**A**) Quantitative RT-PCR analysis of fibroblast, mural cell and hematopoietic markers using total RNA from EC-SP cells (SP), $CD45^+$ hematopoietic cells ($CD45^+$ HC) and $CD31^-CD45^-$ cells (Neg) in the hind limb muscle corrected by expression of the control gene GAPDH. Threshold values for target genes normalized against the level of GAPDH (dCt) are shown. $CD31^-CD45^-$ cells include fibroblasts and mural cells. EC-SP cells do not express the fibroblast marker fibroblast-specific protein 1 (Fsp1), mural cell markers PDGFRβ, NG2 and Desmin, or the hematopoietic marker CD45. (**B**) Results of RT-PCR analysis.

Supplementary Figure S3. Expression of ABC transporters in EC-SP cells.

Quantitative RT-PCR analysis of the ABC transporter genes using total RNA from EC-SP cells (SP) and EC-MP cells (MP). $^{**}P < 0.01$, $^{*}P < 0.05$ (n=4). The expression level of ABCB2 andABCA5 was higher in EC-SP cells than EC-MP cells.

Supplementary Figure S4. Lymphatic ECs do not contain a side population.

(**A**) FACS plots of hind limb $CD45^-$ cells. Cells were analyzed for CD31 and lymphatic marker LYVE1. $LYVE1^+CD31^+CD45^-$ lymphatic ECs are shown in the red gate and $LYVE1^-CD31^+CD45^-$ vascular ECs in the black. (**B**) Hoechst staining of $LYVE1^-CD31^+CD45^-$ vascular ECs [black gate in (**A**)]. EC-SP cells are present in this population. (**C**) Hoechst staining of $LYVE1^+CD31^+CD45^-$ lymphatic ECs [red gate in (**A**)]. EC-SP cells are absent from the lymphatic ECs.

Supplementary Figure S5. Expression of arterial and venous markers in EC-SP cells.

Quantitative RT-PCR analysis of arterial and venous markers using total RNA from EC-SP cells and total ECs that are a mixture of arterial, venous and capillary ECs corrected for the expression of the control gene GAPDH. Expression of the arterial

markers EphrinB2, Neuropilin-1 and Hey1 in EC-SP cells was significantly lower than in total ECs. The venous markers EphB4 and Coup-TFII in EC-SP cells were not significantly different compared to total ECs. Error bars are ± SEM. $^{**}P < 0.01$ (n=4).

Supplementary Figure S6. Cobblestone-like colonies generated by EC-SP cells.

Representative image of a cobblestone-like (sheet-like) colony generated from a single EC-SP cell. Approximately 1.2±0.5% (n=4) of the colonies from EC-SP cells form cobblestone-like colonies on OP9 feeder cells. Scale bar, 200 μm.

Supplementary Figure S7. EC-SP cells in different organs possess colony-forming ability.

EC-SP and EC-MP cells from different organs were cultured on OP9 feeder cells. Quantitative evaluation of the number of colonies expressing CD31 is shown. EC-SP cells in the liver, heart and lung have significantly higher colony-forming ability than EC-MP cells. $^{**}P < 0.01$ (n=5).

Supplementary Figure S8. EC colonies generated by EC-SP cells do express endothelial markers.

EC-SP cells were cultured on OP9 feeder cells and colonies were double-stained with anti-CD31 antibody and for other EC markers, i.e. anti-CD34, -CD105, -Flk1, -VE-cadherin, -vWF or -ZO-1. Merged images from the top two panels are shown at the bottom. Scale bars, 200 μm.

Supplementary Figure S9. Cells generated from EC-SP cells do not express hematopoietic markers.

(**A**) Cells generated from EC-SP cells on OP9 feeder cells were double-stained with anti-CD31 antibody and for hematopoietic markers, i.e., anti-B220, -CD4, -CD8, -Gr1, -Mac1, -Ter119 or –CD45. (**B**) c-Kit^{+}Sca-1^{+}Lin^{-} hematopoietic stem cells from EGFP mice (GFP-KSL) were cultured on OP9 feeder cells as a comparison. Cells were stained with anti-CD45 or -CD31 antibody. Note that hematopoietic cells do not form networks and there are no CD31-positive cells. Scale bars, 200 μm.

Supplementary Figure S10. Development of ECs from EC-SP cells depends on VEGF.

EC-SP cells were cultured on OP9 feeder cells in the presence or absence of VEGFR2-Fc (1 ng/ml). Neutralization of VEGF by VEGFR2-Fc completely blocked the

emergence of EC colonies. Scale bars, 5 mm (upper panels), 500 μm (lower panels).

Supplementary Figure S11. EC-SP cells derived from VE-Cadherin-Cre-ERT2 mice mated with Flox-CAT-EGFP mice reconstitute newly–developed blood vessels.

(**A**) FACS plots of hind limb $CD45^-$ cells derived from mice expressing EGFP under the transcriptional control of the VE-cadherin promoter (VE-cad-GFP mice). Cells were analyzed for CD31 and GFP. $GFP^+CD31^+CD45^-$ cells are shown in the black gate. (**B**) Hoechst staining of $GFP^+CD31^+CD45^-$ cells [black gate in (**A**)]. The percentage of EC-SP cells is comparable to wild-type mice. (**C**) Fluorescent stereomicroscopic image of hind limb muscle 2 weeks after transplantation of EC-SP cells from VE-cad-GFP mice. ECs derived from EC-SP cells generate fine vascular architecture with large lumens connected to the systemic circulation and filled with red blood cells. Scale bars, 50 μm.

Supplementary Figure S12. ECs generated from EC-SP cells do not show endothelial-mesenchymal transition in vitro.

EC-SP cells from EGFP mice were cultured on OP9 feeder cells and after the formation

of the EC colonies, TGFβ1 or TGFβ2 was added to the medium (10 ng/ml and 20 ng/ml respectively). After 48 hours, all the GFP-positive EC colonies were SMA-negative. Note that OP9 feeder cells are SMA-positive and the cell shape of OP9 feeder cells changed after addition of TGFβ. Scale bars, 200 μm.

Supplementary Figure S13. EC-SP cells do not trans-differentiate into the hematopoietic lineage in vitro or in vivo.

(**A**) EC-SP cells or BM KSL cells from EGFP mice were cultured in methylcellulose semi-solid medium for detection of colony forming units in culture (CFU-c). After 7 days, no GFP^+ colonies were visibly derived from EC-SP cells, unlike with KSL cells. (**B**) Gross appearance of spleen from mice transplanted with GFP-EC-SP cells or GFP-KSL cells. Only GFP-KSL transplanted mice formed GFP^+ colonies in the spleen after 12 days. (**C**) FACS plots of BM cells from mice transplanted with GFP-EC-SP cells or KSL cells. EC-SP cells did not contribute to BM hematopoietic cells. Scale bars, 500 μm (**A**) and 2 mm (**B**).

Supplementary Figure S14. EC colonies are generated from single EC-SP cells.

To confirm that all the EC colonies were derived from single ECs, $5x10^2$ EC-SP cells

collected from EGFP mice were mixed with $5x10^2$ EC-SP cells from wild-type mice. They were then cultured on OP9 feeder cells. The colonies were double-stained with anti-CD31 antibody (red) and anti-GFP antibody (green). Note that all colonies are either GFP-positive or negative and no mixed color colonies are seen, suggesting that each EC colony was derived from a single EC-SP cell.

Supplementary Figure S15. EC-SP cells induced by ischemia retain colony-forming ability.

(**A, B**) EC-SP cells or EC-MP cells at day 3 or day 14 after induction of ischemia, or control EC-SP cells in the steady state, were cultured on OP9 feeder layers. The colony-forming ability of the EC-SP cells from ischemia day 3 did not differ significantly from control EC-SP cells and was significantly higher than EC-MP cells from ischemia day 3. The colony-forming ability of day 14 EC-SP cells was not significantly different from the steady state and ischemia day 3. $^{**}P < 0.01$ (n=6).

Supplementary Figure S16. EC-SP cells become mature vessels after transplantation.

EC-SP cells sorted from GFP mouse were transplanted into ischemia-induced hind

limbs of wild-type mice. After 14 days, hind limb muscle was dissected and stained with anti-GFP (**A**), anti-SMA (**B**) and anti-CD31 (**C**) antibodies. Merged image of panels (**A**), (**B**) and (**C**) is presented in (**D**). (**E**), (**F**), (**G**) and (**H**) show higher-power views of the areas indicated by the box in (**A**), (**B**), (**C**) and (**D**), respectively. ECs derived from EC-SP cells are covered with mural cells and connected to GFP-negative ECs. Arrow indicates connection of GFP-positive ECs and GFP-negative ECs. Scale bars, 150 μm (**A-D**) and 50 μm (**E-H**).

Supplementary Figure S17. Quantitative RT-PCR analysis of PHD2 and sFlt1.

Quantitative RT-PCR analysis of PHD2 and sFlt1 using total RNA from EC-SP cells and EC-MP cells. $^{**}P < 0.01$, (n=10). PHD2 expression in EC-SP cells is nearly 45% of that of EC-MP cells, but expression levels of sFlt1 did not differ significantly (n=10). Error bars are ± SEM.

Supplementary Figure S18. Endothelial BrdU retention assay.

(**A**) EC-SP and EC-MP cells sorted from mice which injected with BrdU (100 mg/kg, Sigma) at P3, P4 and P5 daily and maintained for 7 weeks were put onto slides and stained with TO-PRO3 (red) and anti-BrdU (green) antibody. (**B**) Quantitative

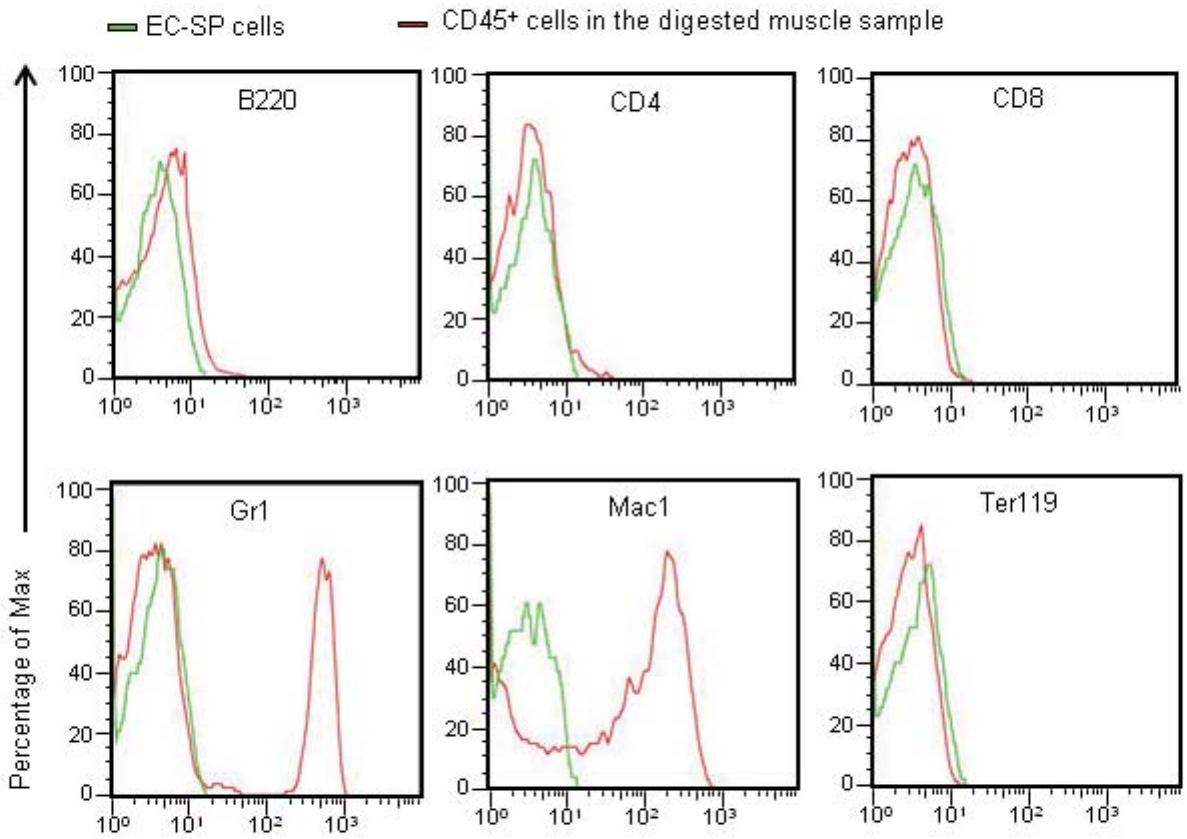
evaluation of the percentage of $BrdU^+$ EC-SP cells and $BrdU^+$ EC-MP cells. Error bars are ± SEM. $^{**}P < 0.01$, (n=15 random low power fields). Although EC-SP cells include about 20% of BrdU-retaining cells (statistically significant compared to EC-MP cells), EC-MP cells also include about 10% of BrdU-retaining cells. 20% of EC-SP cells and 10% of EC-MP cells mean that $BrdU^+$ EC-SP and EC-MP cells are approximately 0.2% and 7% of total ECs, respectively. Scale bars, 50 μm (**A**).

Supplementary Figure S19. Identification of EC-SP cells from ECs in tumor.

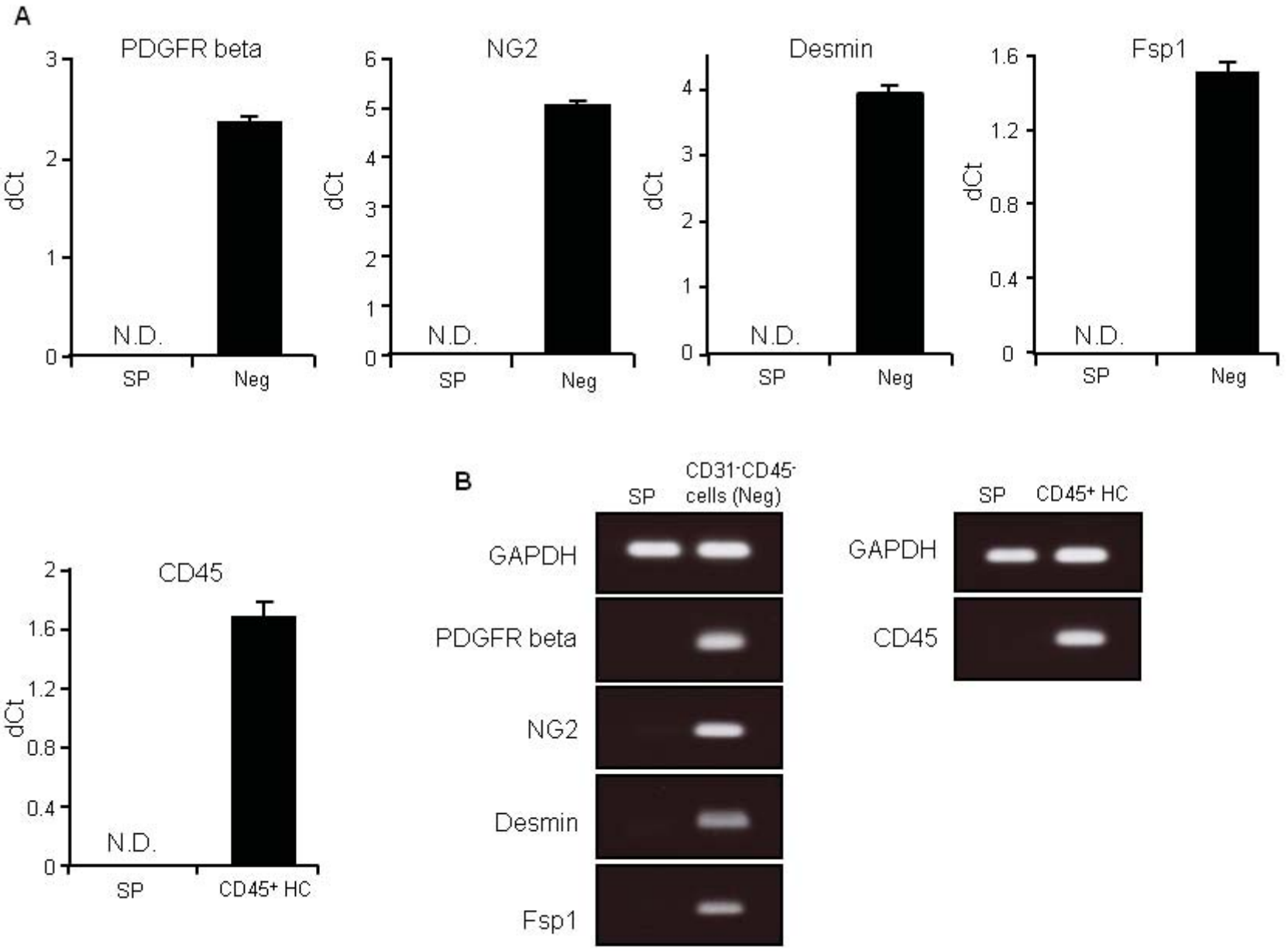
Lewis Lung Carcinoma cells (3×10^6 cells) were inoculated subcutaneously into wild-type mice and after a few weeks, the tumor was minced, enzymatically digested and stained with Hoechst and antibodies. (**A**) The EC-SP population is present at a high percentage in the $CD31^+CD45^-$ EC fraction (4.25±0.38) compared to EC-SP cells residing in normal tissue. Addition of verapamil selectively prevented Hoechst exclusion from EC-SP cells obtained from the tumor. (**B**) Relationship between size of the tumor and the percentage of EC-SP cells is shown. (**C and D**) EC-SP and EC-MP cells from the tumor were sorted and cultured on OP9 feeder cells. After 10 days the colonies were stained with anti-CD31 antibody and the ratio of numbers of colonies were evaluated. Error bars are ± SEM. $^{**}P < 0.01$, (n=6). Scale bars, 500 μm.

Supplementary Movie S1. Time-lapse analysis of an EC-SP cell.

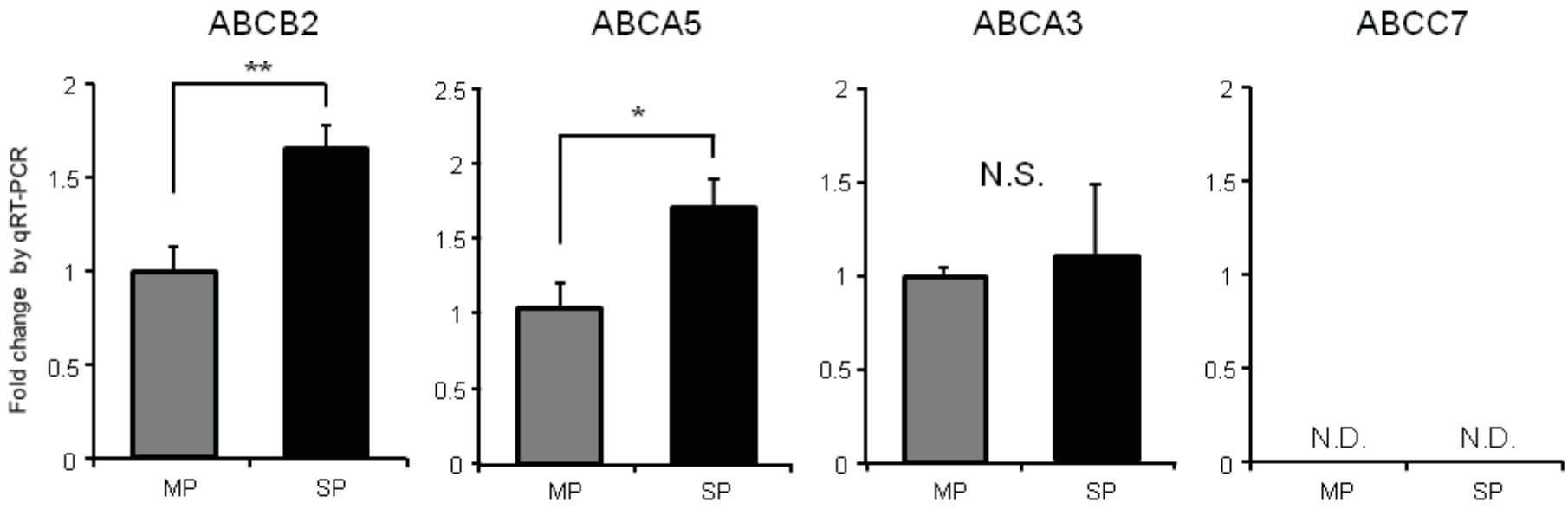
The movie shows an EC-SP cell cultured for 10 days. Scale bar, 100 μm.



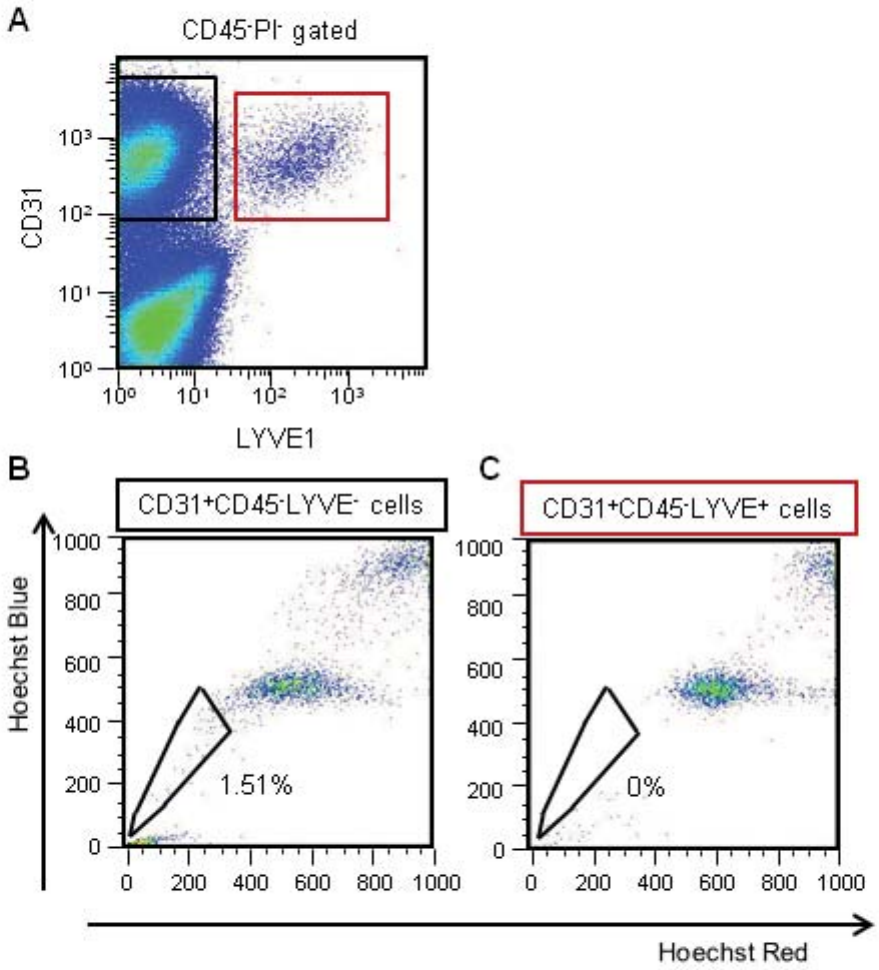
Supplementary Figure S1 Naito H. et al.



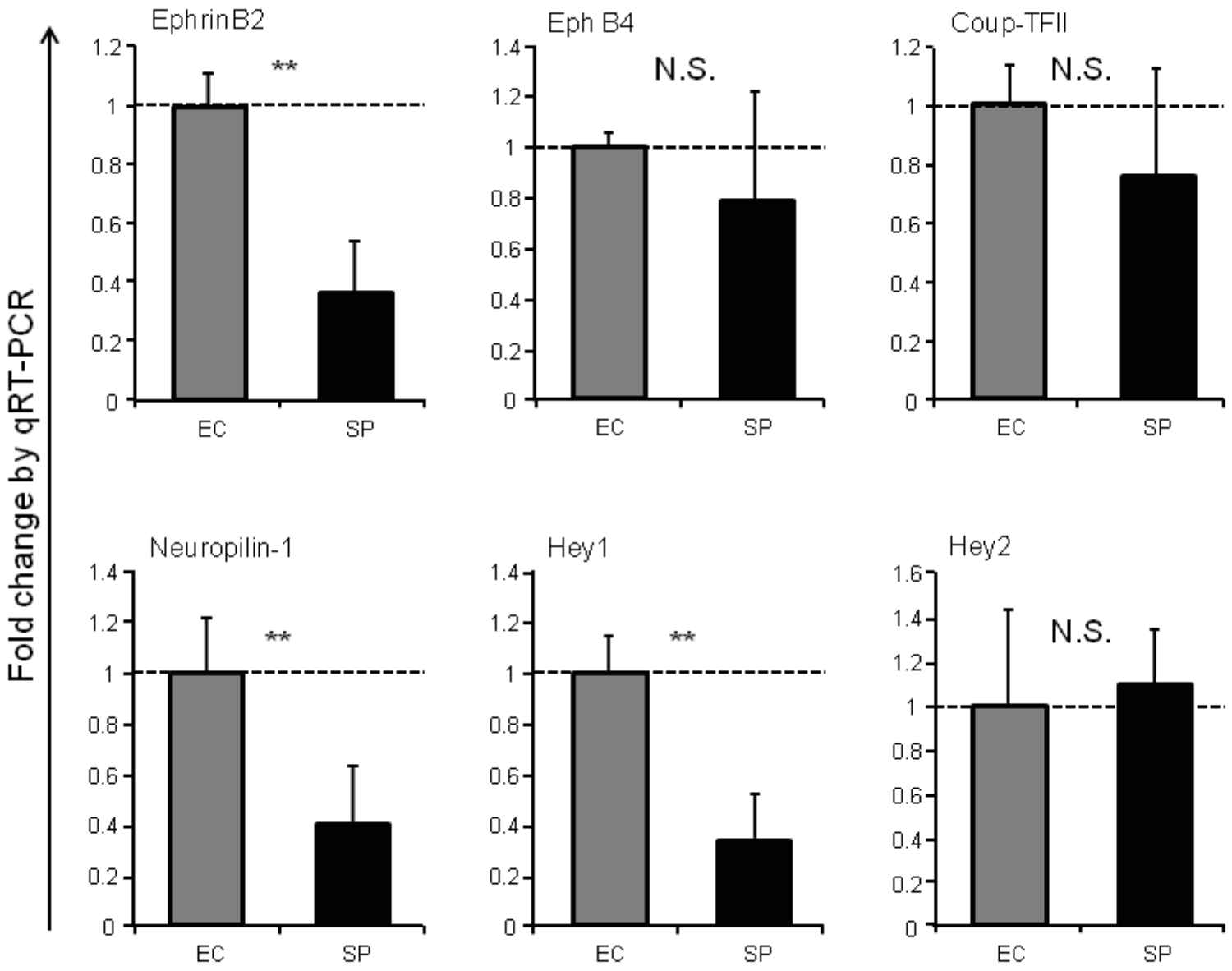
Supplementary Figure S2 Naito H. et al.



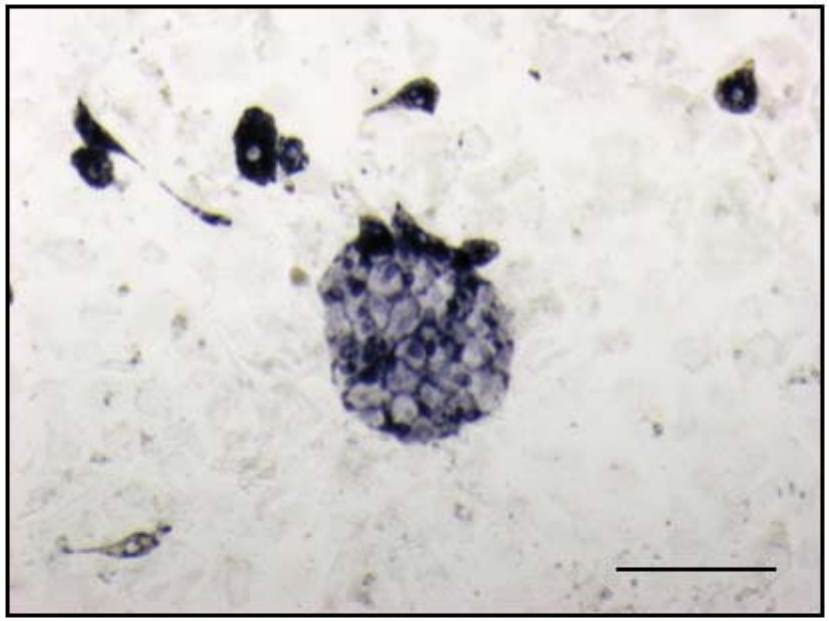
Supplementary Figure S3 Naito H. et al.



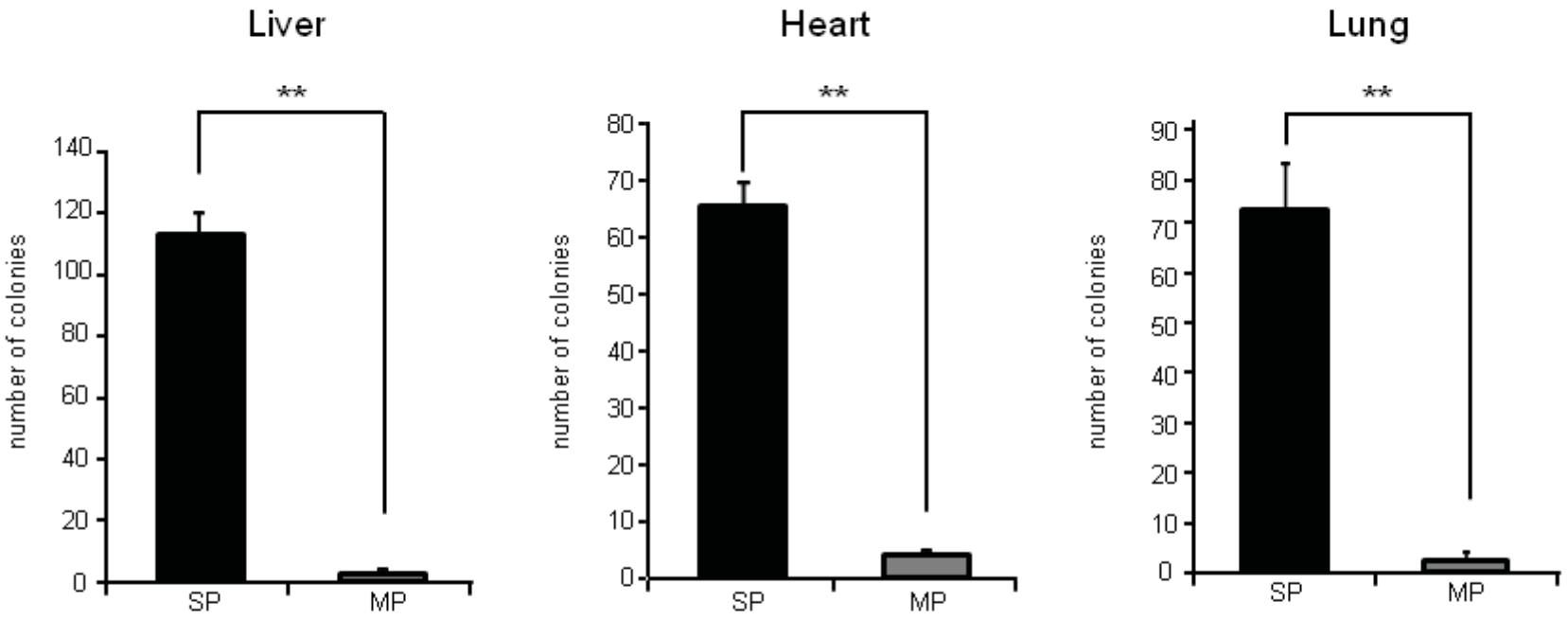
Supplementary Figure S4 Naito H. et al.



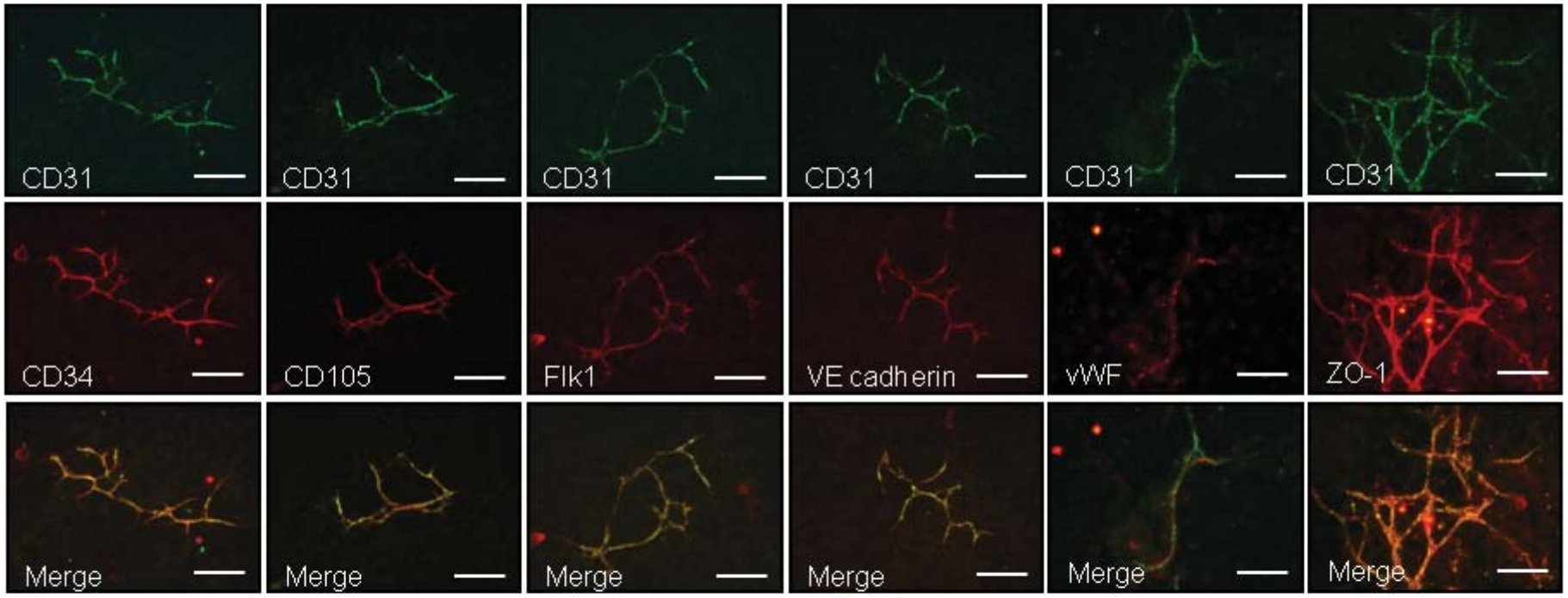
Supplementary Figure S5 Naito H. et al.



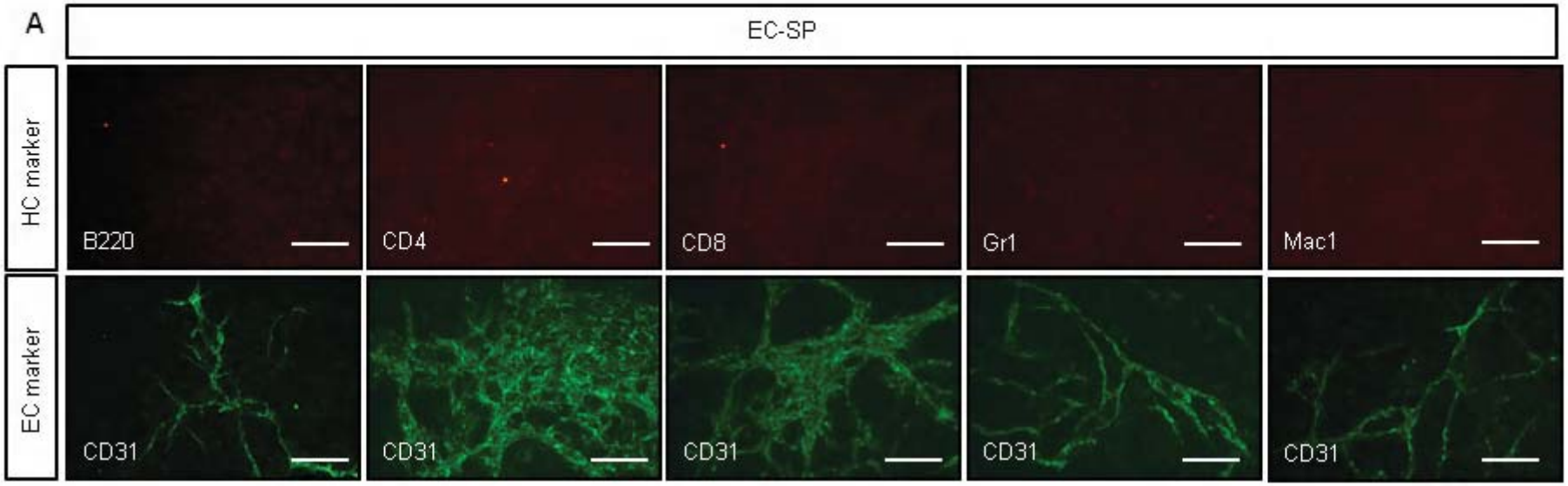
Supplementary Figure S6 Naito H. et al.

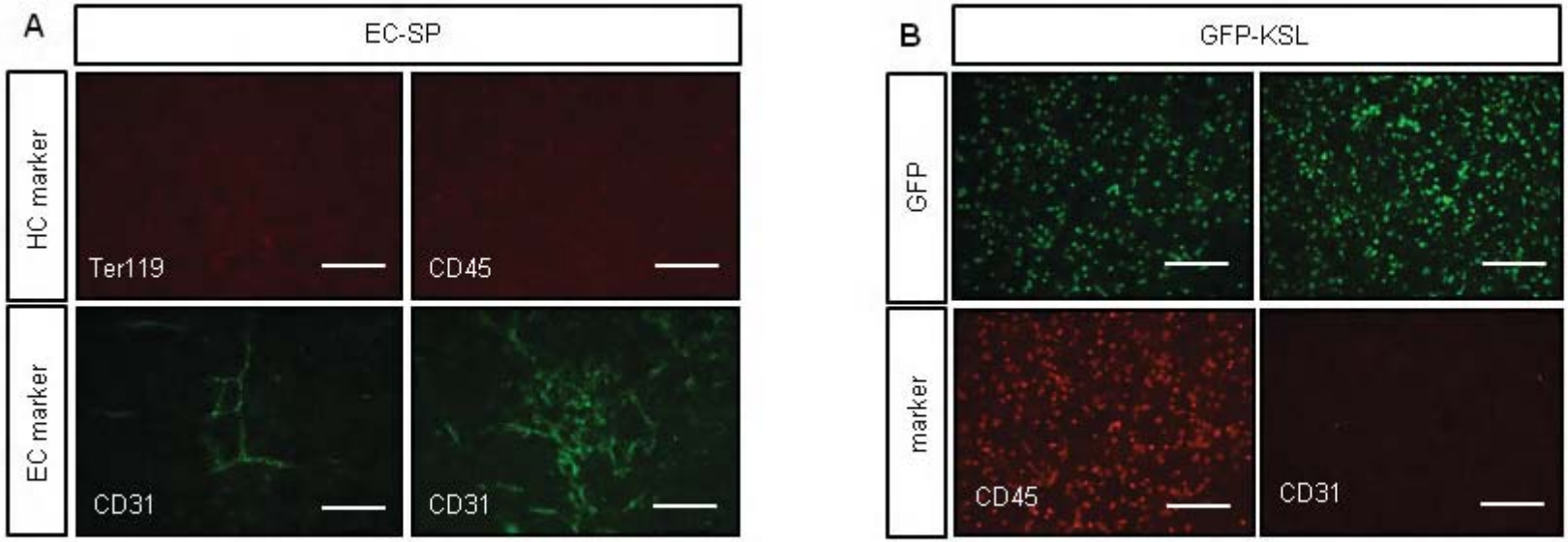


Supplementary Figure S7 Naito H. et al.

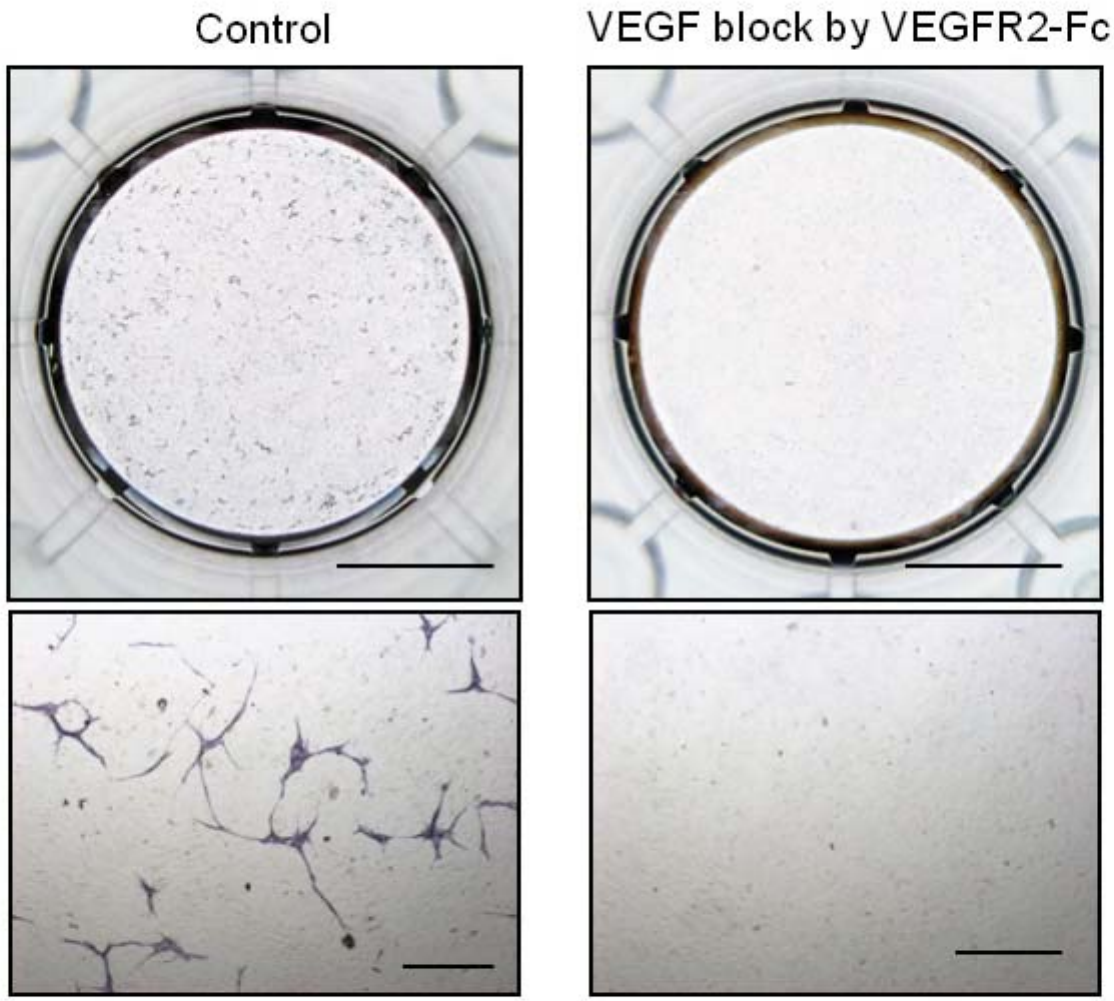


Supplementary Figure S8 Naito H. et al.

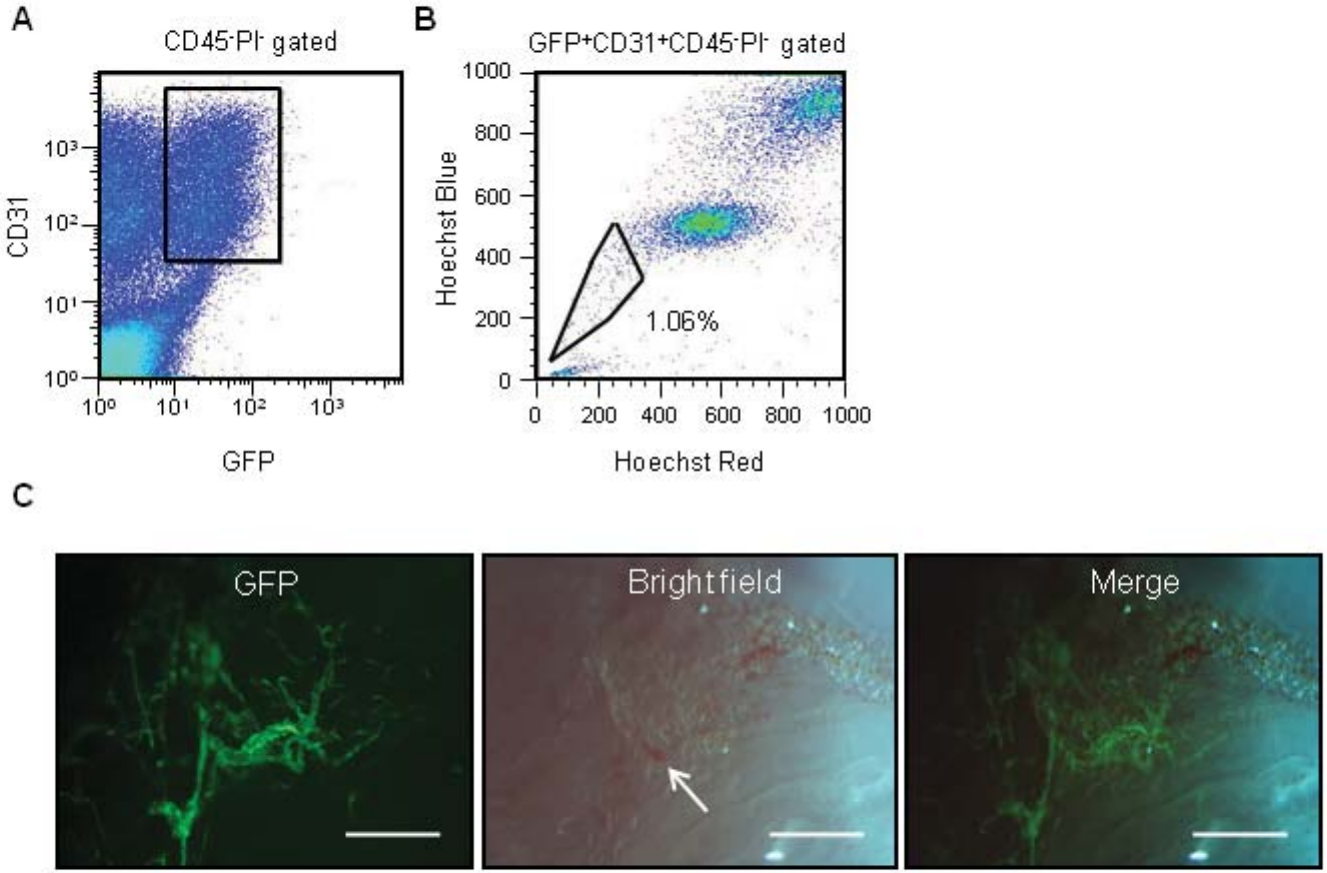




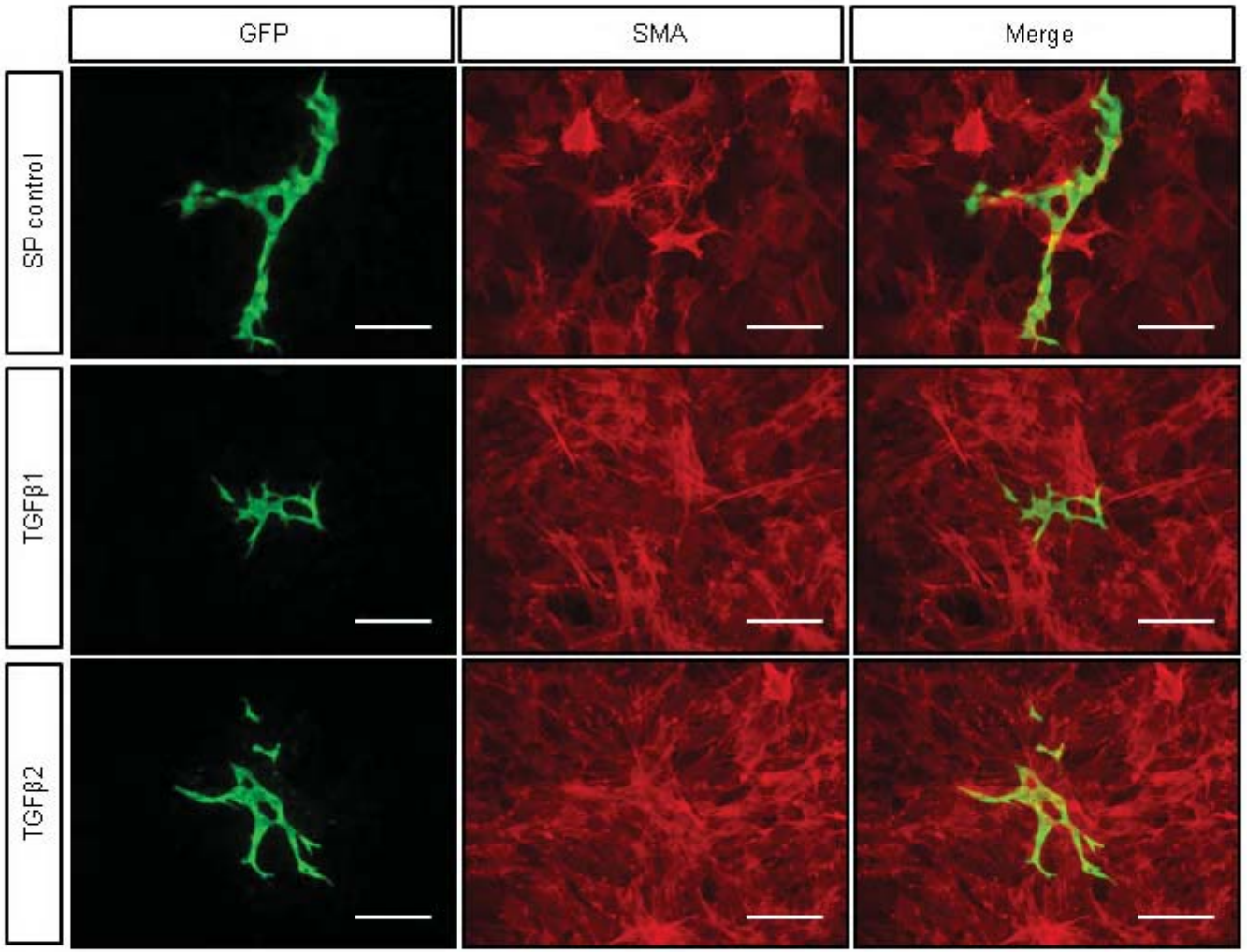
Supplementary Figure S9 Naito H. et al.



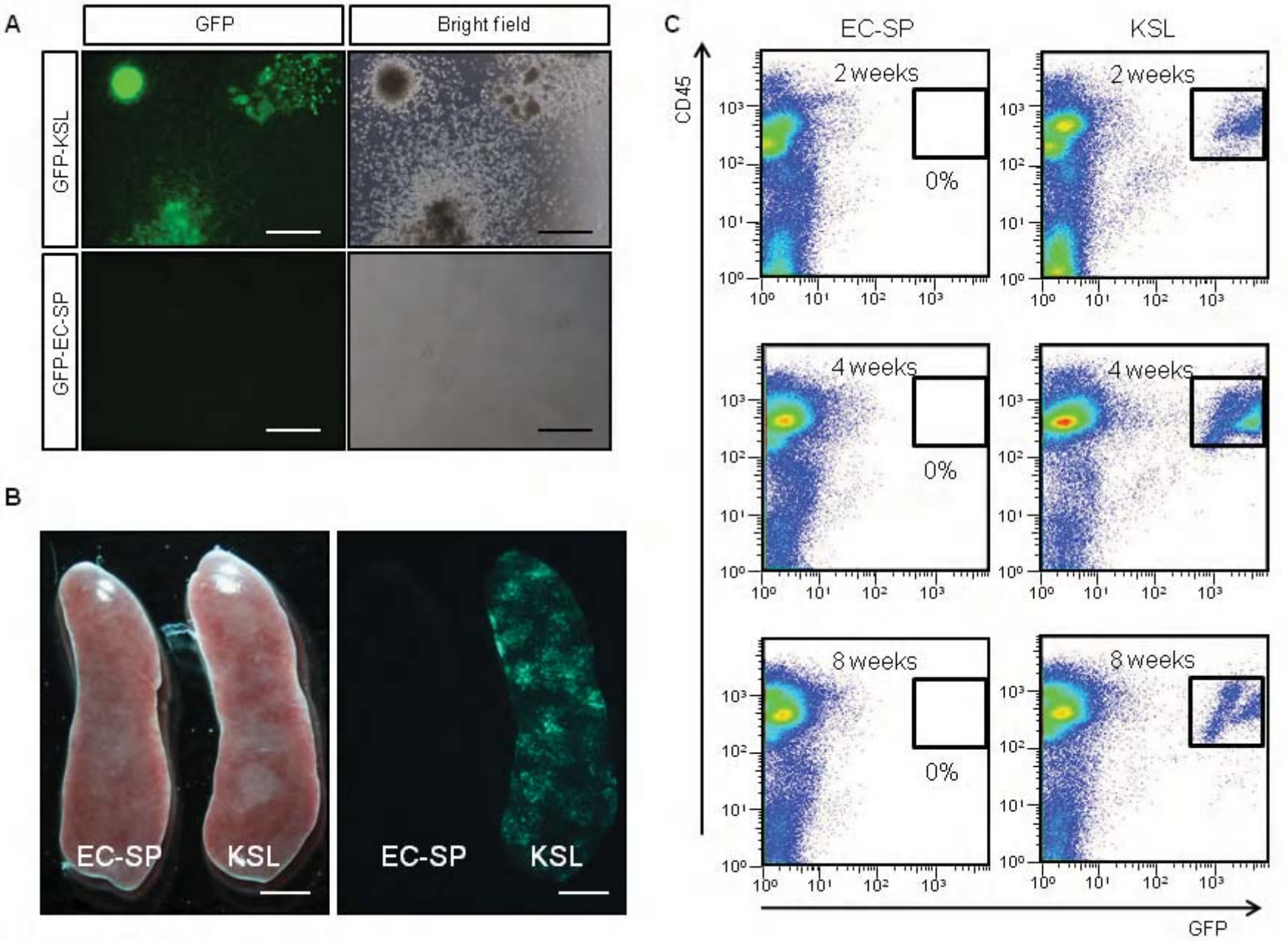
Supplementary Figure S10 Naito H. et al.



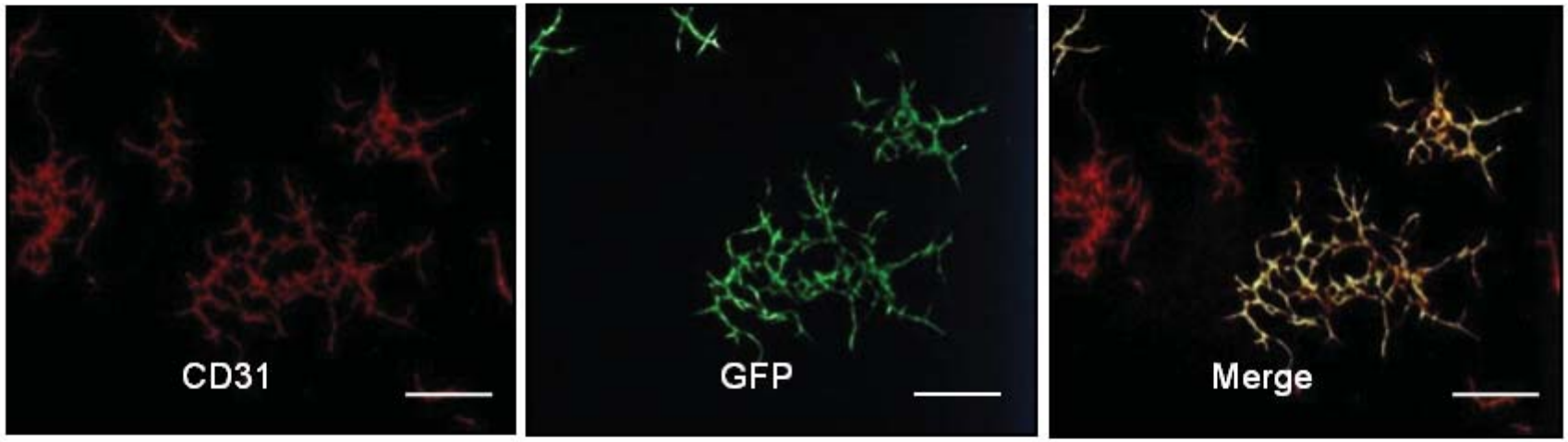
Supplementary Figure S11 Naito H. et al.



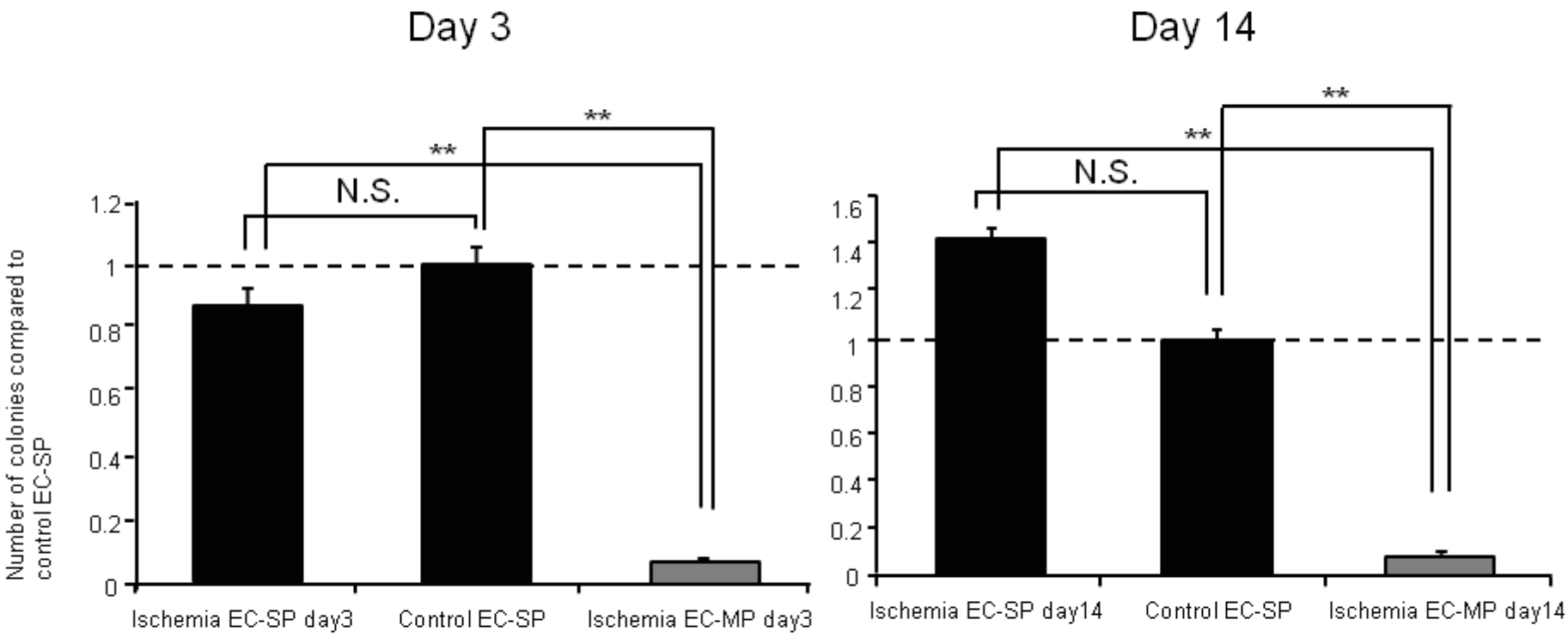
Supplementary Figure S12 Naito H. et al.



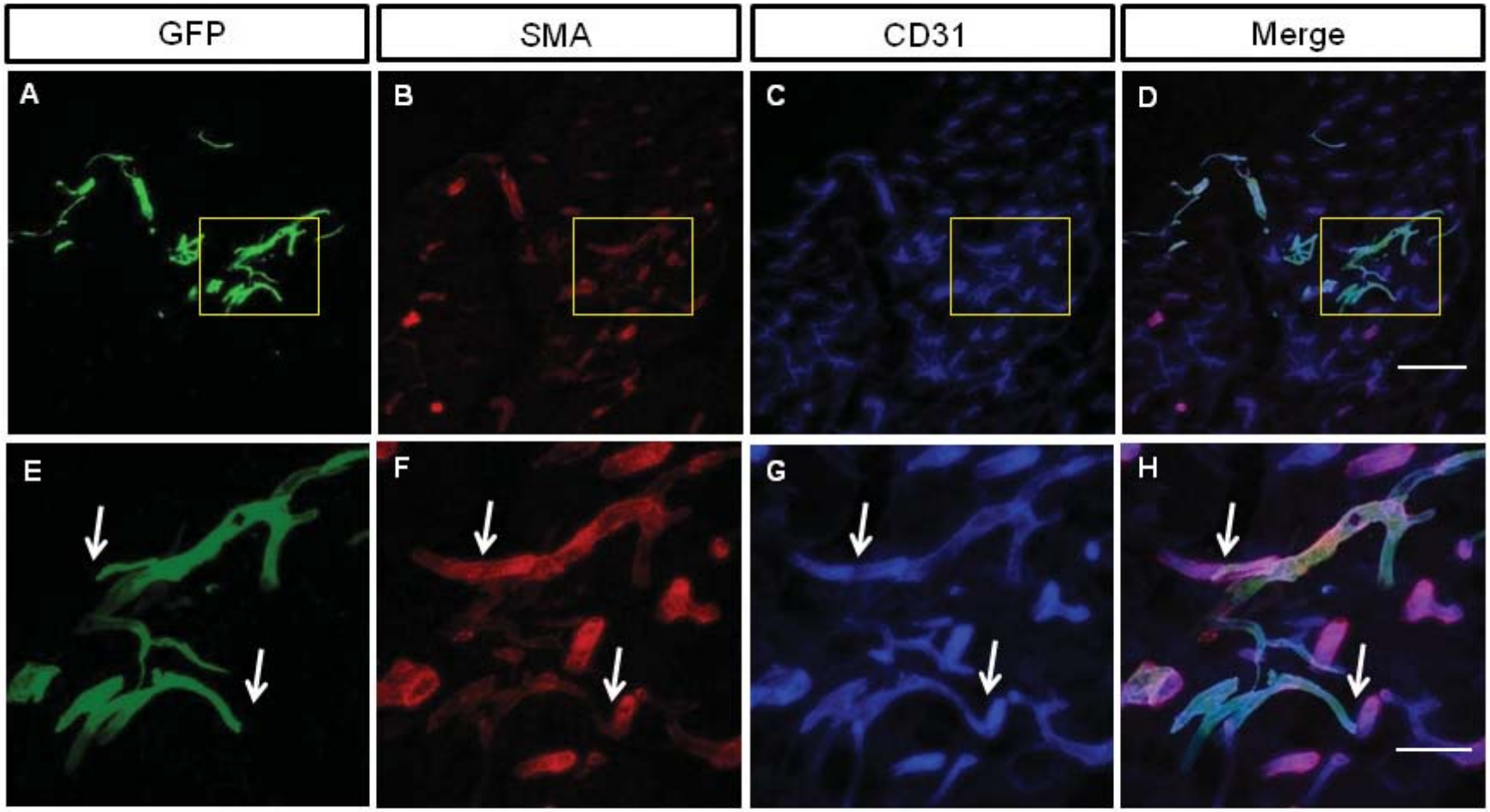
Supplementary Figure S13 Naito H. et al.



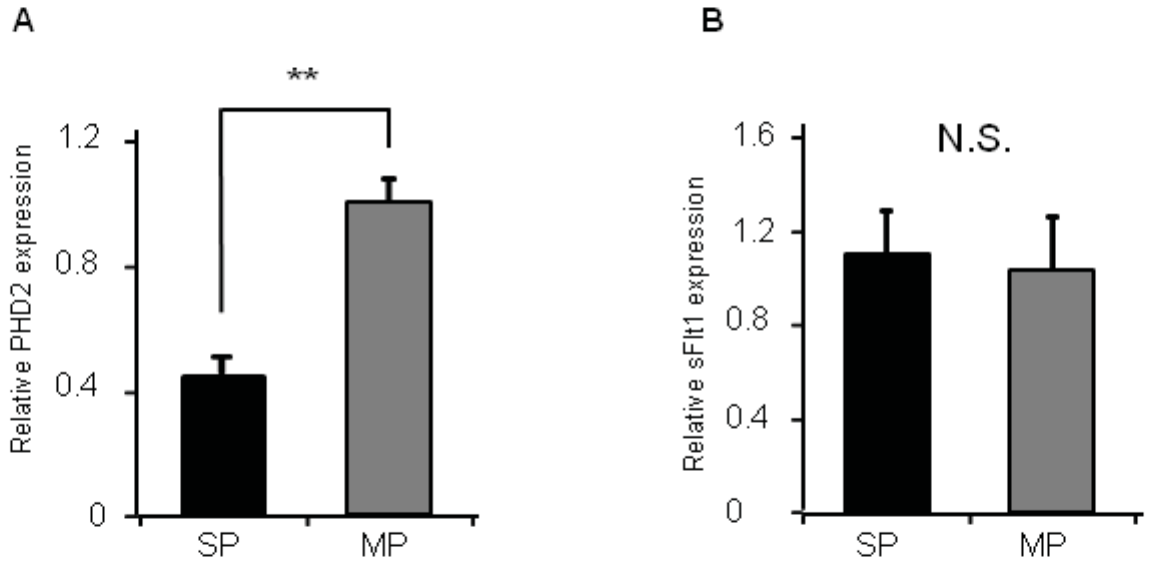
Supplementary Figure S14 Naito H. et al.



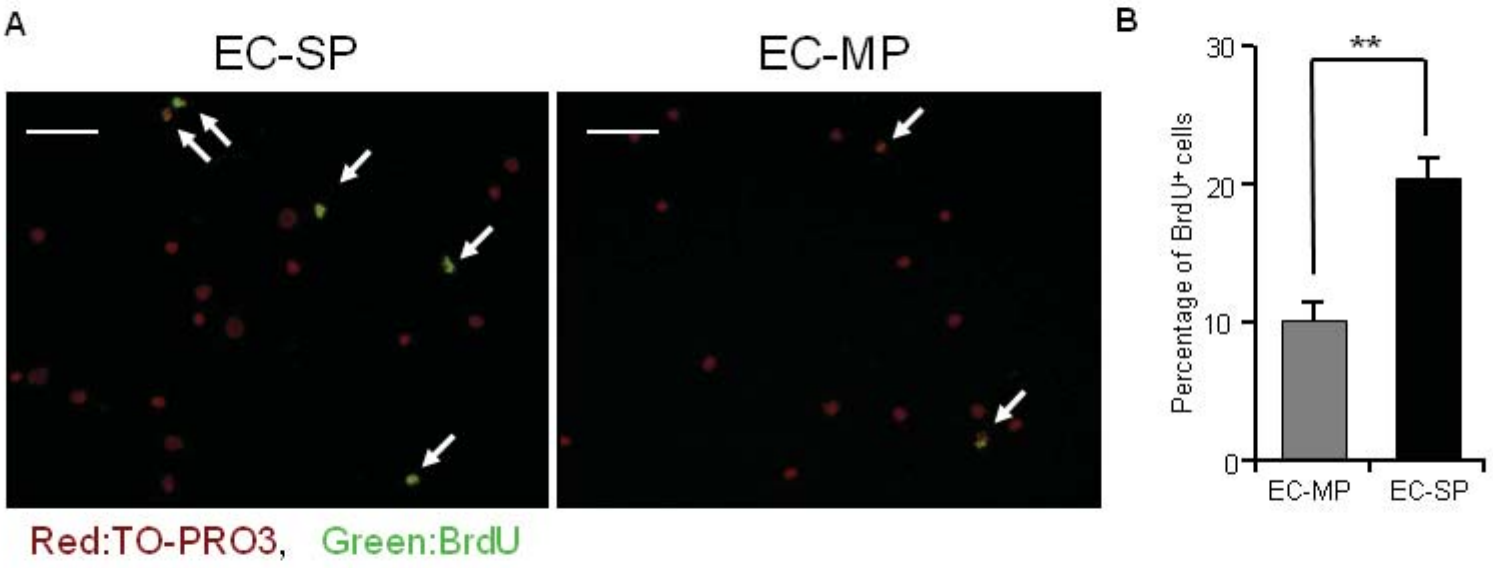
Supplementary Figure S15 Naito H. et al.



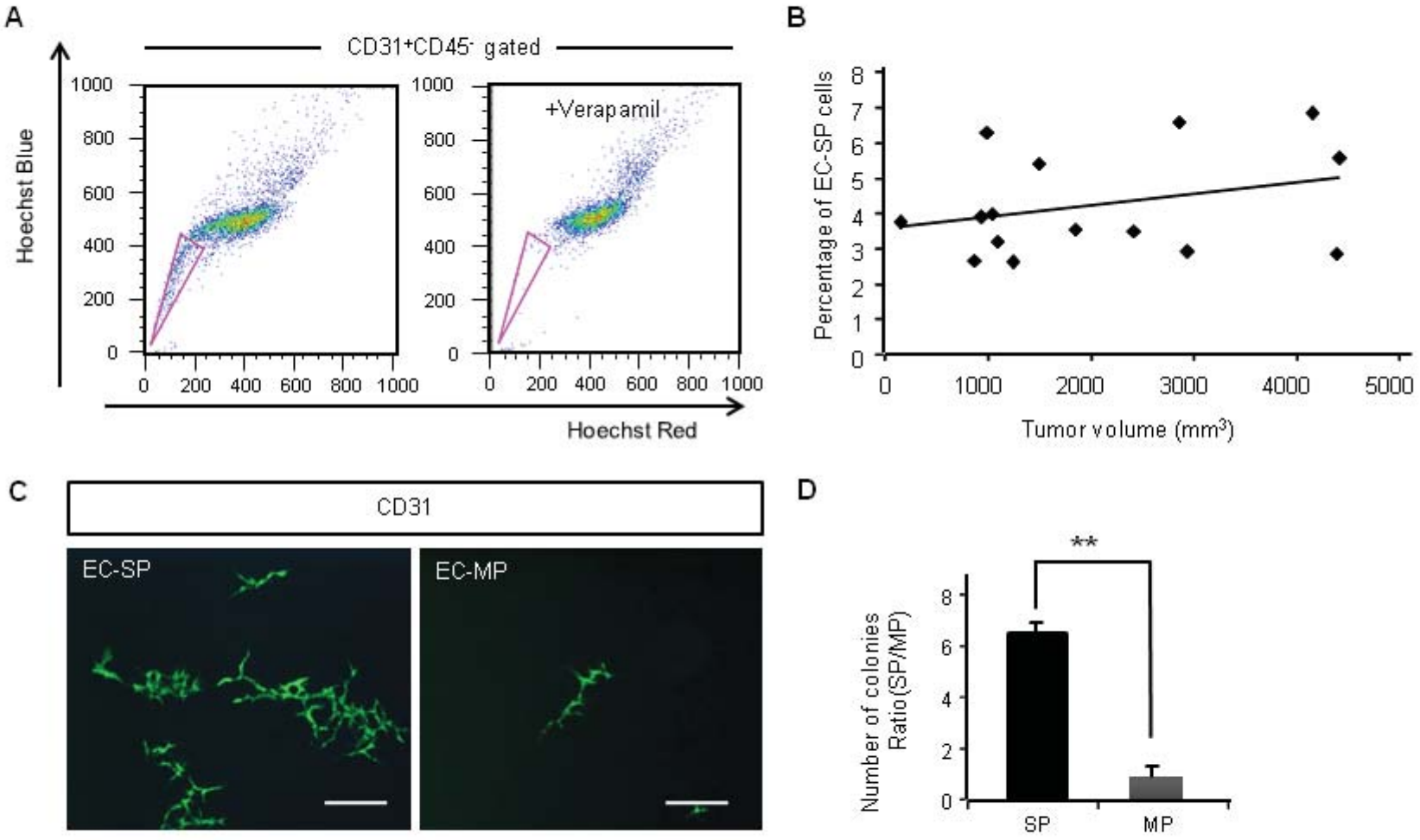
Supplementary Figure S16 Naito H. et al.



Supplementary Figure S17 Naito H. et al.



Supplementary Figure S18 Naito H. et al.



Supplementary Figure S19 Naito H. et al.

Supplementary Table SI
Oligonucleotide sequences for PCR

Gene	Primer sequence	Gene	Primer sequence
VEGFR2	5'-CCCAGCATCTGGAAATCCTA-3' 5'-CCGGTTCCCATCTCTCAGTA-3'	Pacsin1	5'-ACCACTGCCAAGAAGGAGAA-3' 5'-CACTCGGTGGCATATGTTTG-3'
Tie2	5'-AGTCCGAGCTAGAGTCAACACC-3' 5'-ACCTTATACCGGATGATGATGG-3'	Tnfrsf11b	5'-GACACAGTCCACAAGAGCAAAC-3' 5'-CAGAGGTCAATGTCTTGGATGA-3'
VE-cadherin	5'-CCCGTCTTTACTCAATCCACA-3' 5'-ATCTGGGTCCACAACAGTCAG-3'	CDCa8	5'-TCAAAAGATGCCTTCCATCC-3' 5'-CTCATAGCTGGCGTCACAAA-3'
Sca1	5'-GTTACTCAGGAGGCAGCAGTTATT-3' 5'-GGAAGTCTTCACGTTGACCTTAGT-3'	E-selectin	5'-GTCCCTTGGTAGTTGCACTTTC-3' 5'-CGTTGTAAGAAGGCACATGGTA-3'
EphB4	5'-AATGTCACCACTGACCGTGA-3' 5'-TCAGGAAACGAACACTGCTG-3'	Glycam1	5'-AATGAAGACTCAGCCCACAGA-3' 5'-CCTTGGAAAGGTCCTTACTGG-3'
EphrinB2	5'-GTTGGACAAGATGCAAGTTCTG-3' 5'-TCTCCCATTTGTACCAGCTTCT-3'	Multimerin	5'-CAGCAGTGGACTCTGTTCCA-3' 5'-TCAGCCTGGTTACTGTGTGC-3'
Notch1	5'-TGTTGTGCTCCTGAAGAACG-3' 5'-GTGGGAGACAGAGTGGGTGT-3'	Reelin	5'-CCATACTGTGGCCATGACTG-3' 5'-CACCTGGTTGTCCATGTGAG-3'
Notch4	5'-TAATCCCTGCCTGAACCAAG-3' 5'-GGGGTCACTCAGACATTCGT-3'	CD44	5'-TATCCTCGTCACGTCCAACA-3' 5'-TCCATCGAAGGAATTGGGTA-3'
CXCR4	5'-TAGGATCTTCCTGCCCACCAT-3' 5'-TGACCAGGATCACCAATCCA-3'	Timp4	5'-AGGGAGAGCCTGAATCATCA-3' 5'-ATAGAGCTTCCGTTCCAGCA-3'
ABCB1a	5'-CCAGCAGTCAGTGTGCTTACA-3' 5'-GCCACTCCATGGATAATAGCA-3'	PHD2	5'-AGCGAGCGAGAGCTAAAGTAAA-3' 5'-GACGTCTTTGCTGACTGAATTG-3'
ABCG2	5'-CCAGCAGTCAGTGTGCTTACA-3' 5'-GCCACTCCATGGATAATAGCA-3'	sFlt1	5'-GAAGACATCCTTCGGAAGCACGAA-3' 5'-TTGGAGATCCGAGAGAAAATGG-3'
HIF1a	5'-TCAAGTCAGCAACGTGGAAG-3' 5'-TATCGAGGCTGTGTCGACTG-3'	GAPDH	5'-TGGCAAAGTGGAGATTGTTGCC-3' 5'-AAGATGGTGATGGGCTTCCCG-3'
ABCB2	5'-CTCTTGCCTTGGGGAAATG-3' 5'CTGTGCTGGCTATGGTGAGA-3'	ABCC7	5'-GACACTTTGCTTGCCCTGAG-3' 5'-AAGAATCCCACCTGCTTTCA-3'
ABCA5	5'-TTCTATGTCCTCCTGGCTGTG-3' 5'-TGACCAATACGATGGCTTCA-3'	ABCA3	5'-TTATGCCCTCCTACTGGTGTG-3' 5'-CTTGTCCTTATTGCCCACTTG-3'
PDGFRβ	5'-CCGGAACAAACACACCTTCT-3' 5'-TATCCATGTAGCCACCGTCA-3'	CD45	5'-TTTCGCTACATGACTGCACAC-3' 5'-CGAAGGTTGTCCAACTGACAT-3'
NG2	5'-AGCAAGGAAGTGCAGAGGAG-3' 5'-CAGAGGACATCTCGTGCTCA-3'	Desmin	5'-GCGGCTAAGAACATCTCTGA-3' 5'-TCCCTCATCTGCCTCATCA-3'
Fsp1	5'-TCAGCACTTCCTCTCTCTTGG-3' 5'-TGAACTTGTCACCCTCTTTGC-3'	COUP TFII	5'-CGGAGGAACCTGAGCTACAC-3' 5'-CAGGTACGAGTGGCAGTTGA-3'
Hey1	5'-TGAGCTGAGAAGGCTGGTAC-3' 5'-ACCCCAAACTCCGATAGTCC-3'	Hey2	5'-TGAGAAGACTAGTGCCAACAGC-3' 5'-TGGGCATCAAAGTAGCCTTTA-3'
Np-1	5'-ATGGGTGGACTCCAGGAGAA-3' 5'-GATCCAGTCCTCTCCGTTGG-3'	αSMA	5'-CTGAAGAGCATCCGACACTG-3' 5'-AGAGGCATAGAGGGACAGCA-3'