SUPPLEMENTAL MATERIAL

Semaphorin 3A overcomes cancer hypoxia and metastatic dissemination induced by anti-angiogenic treatment in mice

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SUPPLEMENTAL METHODS

Determination of RIP-Tag2 and HPV16/E_2 tumor burden and immunofluorescence analysis

Pancreas, LNs and livers from RIP-Tag2 mice and uterine cervices, lungs and livers from K14-HPV16/E_2 were dissected and collected. Macroscopic tumors (≥ 1 x 1 mm) were excised, measured with a caliper and processed as described previously (1, 2). Tissues were fresh frozen in OCT or fixed in buffered formalin and embedded in paraffin. For the histopathology analysis of cervical cancer, 10-μm-thick paraffin sections were serially sectioned using a Leica 2135 microtome were deparaffinized and subjected to graded rehydration through xylene, 100%, 95%, 70% ethanol and then stained with H&E at intervals of 100 μm. The characterization of neoplastic stages has been previously reported (3). Tumor volume was determined using the following formula ($V = 2/3 \times A \times Z$), where A is the cross-sectional area of the tumor and Z is the depth of tumor calculated through serial sections. Frozen tissues were used for immunofluorescence staining. Cryostat sections (10 μm) were air-dried, fixed in zinc-fixative (6,05g Tris, 0,35g Ca(C2H3O2)2, 2,5g Zn(C2H3O2)2, 2,5g ZnCl, 3,8 ml HCl 37%) for 10 minutes and blocked in 3% BSA and 5% donkey serum in 1× PBS.

Tissues were stained by employing the following primary antibodies: purified rat monoclonal anti-Panendothelial Cell antigen (Meca32) (550563, clone Meca32, 1:100, BD Pharmingen); rabbit polyclonal anti-NG2 (Chondroitin sulphate proteoglycan, 1:100, AB5320, Chemicon); rabbit polyclonal anti-αSMA (AB5694, 1:100, Abcam); goat polyclonal anti-PDGFR-β (AF1042, 1:20, R&D); rabbit polyclonal anti-Desmin (AB907, 1:20, Chemicon); rabbit polyclonal anti-SV40 T-antigen (sc-20800, 1:50 Santa Cruz); rabbit polyclonal anti-HPV16-E7 protein (250629, 1:100, Abbiotec); rat monoclonal anti-E-cadherin (13-1900, 1: 100, Invitrogen); rabbit polyclonal anti-Vimentin (ab45939, 1:100, Abcam); goat polyclonal anti-Met (AF527, 1:20, R&D); rabbit monoclonal anti-phospho-Met (3077, 1:50, Cell Signaling), rabbit polyclonal anti-c-Myc (PRB-150P, 1:200, Covance). The secondary antibodies used were: anti-Rabbit Alexa Fluor-488 and Alexa Fluor-555; anti-Rat Alexa Fluor-488; anti-Fluorescein/Oregon Green Alexa Fluor- 488 and anti-Goat Alexa Fluor-488 and Alexa Fluor-555 (1:400, Molecular Probes). Nuclei were counterstained with DAPI (Invitrogen). All immunofluorescence images were captured by using a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems) and by maintaining the same laser power, gain and offset settings. All immune-localization experiments were performed on multiple tissue sections and included negative controls for determination of background staining, which was negligible.

Analysis of tumor vasculature

Tumor vasculature has been evaluated as previously described (4). For each animal, the total vessel area in each tumor section of at least five 200x power field pictures was quantified as Meca32 positive structures by computer assisted analysis employing Image-ProPlus 6.2 software.

Western blot and protein levels quantification

Tumor islets from the different treatment groups were microdissected from the excised pancreas, to remove the surrounding exocrine tissue and snap frozen (6 mice per group). Proteins were obtained using TriReagent (Sigma-Aldrich) according to manufacturer's instructions. Proteins were quantified using the BCA assay reagent (Thermo scientific). The lysates were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences). After blocking with 3% BSA in 1xTBS, membranes were incubated with primary Ab for 1 hour at room temperature (RT) or overnight at 4°C. We used the following Abs: rabbit polyclonal anti-HIF-1α (AB3883, 1:1000, Chemicon); goat polyclonal anti-CA9 (AF2344, 1:500, R&D); rabbit polyclonal anti-Snail1 (sc-10432, 1:1000, Santa Cruz); mouse monoclonal anti-Vimentin (MAB3400, 1:1000, Millipore); mouse monoclonal anti-E-cadherin (610182, 1:1000, BD Biosciences); rabbit polyclonal anti-N-cadherin (ab18203, 1: 500, Abcam); rabbit monoclonal anti-NF-*k*b (4764, 1:1000, Cell Signaling) and goat polyclonal anti-Vinculin (sc-7649, 1:1000, Santa Cruz). After incubation with a secondary peroxidase-conjugated anti-goat (305-035-003, Jackson Immmuno Research, diluted 1:15000), or anti-rabbit (111-035-003, Jackson Immmuno Research, diluted 1:10000) and anti-mouse (115-035-003, Jackson Immmuno Research, diluted 1:15000), proteins were detected with ECL (Western Lightning Plus, Perkin-Elmer). Chemiluminescent signal was measured and relative levels of protein expression were normalized to vinculin. The relative intensity of the signal of each protein band was determined by means of Bio-Rad Chemi-Doc and Quantity One Program by following the manufacturer's instructions.

Immunoprecipitation

RIP-Tag2 mice were perfused with 1xPBS and tumors were isolated and lysed in Tris-HCl 50 mmol/L pH7.4, NaCl 150 mmol/L, Na3VO4 5 mmol/L, PMSF 1 mmol/L, ZnCl2 0,01 mmol/L, proteases inhibitors, Triton X-100 1%. After centrifugation, protein concentration was determined by the BCA assay reagent. Equal amounts (200μg) of proteins from the homogenate tissue were pre-cleared with protein G agarose (Invitrogen) before incubating with the primary antibodies. Then the supernatant was incubated for 2 hours at 4°C in the lysis buffer containing Protein G agarose, the goat anti-total Met (AF527, 1μg, R&D) or the rabbit monoclonal anti-phospho-Met (3077, 1μg, Cell Signaling) or goat polyclonal anti-Vinculin (1μg, Santa Cruz, Sc-7649, as immunoprecipitation control) antibodies. Immunoprecipitated proteins were boiled in loading buffer with β-2 mercaptoethanol and run on a 8% SDS-polyacrylamide gel. Proteins were blotted on membrane and probed with the goat anti-total Met diluted 1:1000 or the rabbit anti-phospho-Met diluted 1:1000 or the goat anti-vinculin 1:1000 in 3% BSA in 1xTBS. Secondary antibodies horseradish peroxidase-conjugated anti-goat and anti-rabbit respectively were added and the blot was developed with ECL reagents. Relative levels of protein expression were measured as described above.

SUPPLEMENTAL REFERENCES

1. Parangi S, Dietrich W, Christofori G, Lander ES, Hanahan D. Tumor suppressor loci on mouse chromosomes 9 and 16 are lost at distinct stages of tumorigenesis in a transgenic model of islet cell carcinoma. *Cancer Res.* 1995;55(24):6071-6076.

2. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest.* 2003;111(9):1287-1295.

3. Giraudo E, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J Clin Invest.* 2004;114(5):623-633.

4. Maione F, Molla F, Meda C, et al. Semaphorin 3A is an endogenous angiogenesis inhibitor that blocks tumor growth and normalizes tumor vasculature in transgenic mouse models. *J Clin Invest.* 2009;119(11):3356-3372.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Sema3A impairs tumor invasiveness and reduces peri-pancreatic lymph node and liver metastasis induced by sunitinib. (**A**) Histological analysis by means of H&E staining to determine tumor invasion and metastasis incidence in RIP-Tag2 mice treated for 4 weeks (from 12 to 16 weeks of age) with sunitinib (SUN), sunitinib+AAV8-Sema3A (SUN+Sema3A) and AAV8-Sema3A (Sema3A) compared with control. SUN-treated tumors displayed an invasive front extensively intercalated is into the surrounding tissue; islands of normal exocrine tissue were visible inside the tumor, compared with controls (black line). In contrast, Sema3A- and SUN+Sema3A -treated tumors showed a less invasive phenotype in which the tumor tissue is well separated from the surrounding exocrine tissue. (**B,C**) Histological analysis of peri-pancreatic lymph node (LN) and liver metastasis revealed the presence of enlarged LN and liver infiltrated by tumor cells in SUN-treated mice (arrows), compared with controls. LN and liver metastasis were absent or reduced in mice treated with Sema3A or with SUN+Sema3A, respectively. Images are representative of serial sections analysis of tissues per mouse from a total of 30 mice per treatment group. M: metastasis T: tumor; Ac: acinar tissue. Scale bars: 50 μm.

Supplemental Figure 2. Sema3A decreases basal tumor hypoxia in RIP-Tag2 mice. Tumor hypoxia was assessed by means of Pimonidazole adducts immunostaining (arrows) in serial sections of tumors from 12-weeks-, 14-weeks-old RIP-Tag2 mice and from RIP-Tag2 treated for 4 weeks with Sema3A. Sema3A treatment abrogated tumor hypoxia observed in control mice both at the beginning and the end of the therapeutic trial. Images are representative of serial sections analysis of tissues per mouse from a total of 10 mice per treatment group. Scale bars: 50 μm

Supplemental Figure 3. **Sema3A alone or combined with sunitinib enhances tumor tissue perfusion and reduces blood vessel leakage**. (**A-D**) Vessel perfusion and permeability were assessed by means of confocal microscopy analysis and by FITC-lectin (**A**) and FITC-70KDa dextran extravasation (**B**) immunostaining, respectively, in SUN-, SUN+Sema3A - and Sema3A-treated tumors, compared with controls. (**C**) Bar graph shows the percent of FITC-lectin-perfused blood vessels (green) on total area. SUN+Sema3A treatment enhanced vessel perfusion by 81% compared to SUN-treated insulinomas. Sema3A improved vessel perfusion by 38% compared with controls. (**D**) Bar graph shows the percent of FITC-dextran extravasation (green) on total area. SUN+Sema3A treatment reduced vessel permeability by 87% compared with SUN-treated insulinomas. Sema3A alone decreased dextran extravasation by 90% compared with controls. Values are mean ± SD of 5 fields per tumor per mouse from a total of 12 mice per treatment group. Unpaired Mann-Whitney U-test (**$p<0.01$). Scale bars: 50 μm.

Supplemental Figure 4. **Sema3A combined with sunitinib halts the invasiveness and induces highly perfused, pericyte covered and non leaky vasculature in a survival trial**. (**A**) SV40 T-antigen (T-Ag) immunostaining was used to determine the degree of invasiveness in tumors of RIP-Tag2 mice survived 18 weeks after the initial treatment with SUN+Sema3A. All the tumors analyzed were mainly IT or IC1 carcinomas, while no fully invasive (IC2) tumors were found (% of total tumor per animal: IT=60%, IC1=40%, IC2=0%). (**B**) Tumor hypoxia was assessed by means of pimonidazole adducts immunostaining in serial sections of tumors from RIP-Tag2 mice of the survival trial treated with SUN+Sema3A. No hypoxia was detected in all tumors analyzed. (**C**) The pericyte coverage was evaluated by confocal microscope analysis to evaluate the co-localization of Meca32 (green) with α-SMA (red). Highly pericyte vessel coverage has been detected in insulinomas 18 weeks after the initial treatment with SUN+Sema3A. (**D-E**) Vessel perfusion and permeability were assessed by means of confocal microscopy analysis and by FITC-lectin (**D**) and

FITC-70KDa dextran extravasation (**E**) immunostaining, respectively, in SUN+Sema3A -treated RIP-Tag2 tumors. This analysis revealed that the blood vessels of tumors treated with SUN+Sema3A were highly perfused (**D**) and poorly permeable (**E**). Images are representative of serial sections analysis of tissues per mouse from a total of 4 mice analyzed. Scale bars: 50 μm

Supplemental Figure 5. AAV8-Sema3A efficiently targets and expresses Sema3A within the cervix of HPV16/E_2 mice. Sema3A-c-Myc-Tag immunostaining in serial sections of cervical tumors derived from 6 months (6m) old HPV16/E_2 mice infected with AAV8-Sema3A for 4 weeks (**B**), compared with 6m-old HPV16/E_2 animals infected with AAV8-LacZ (control) (**A**). (**C**) Magnification of tumor and transformation zone of HPV16/E_2 infected mice. Arrows indicate Myc-Tag immunostaining in tumor cells (T), epithelial cells of CIN3 lesions (E), columnar cells (C) and in some cells of the stroma (S). To achieve specific gene delivery to the cervix, we established a novel route of administration by injecting the recombinant AAV8 virus expressing either LacZ (for control purposes, not shown) or Sema3A-myc in the distal portion of the abdominal aorta just before its bifurcation into the two common iliac arteries (see *Methods*).

Supplemental Figure 6. **Sema3A alone or combined with sunitinib improves tumor tissue perfusion and decreases blood vessel permeability in HPV16/E_2 mice.** (**A-D**) Vessel perfusion and permeability were assessed by means of confocal microscopy analysis and by FITC-lectin (**A**) and FITC-70KDa dextran extravasation (**B**) immunostaining, respectively, in SUN-, SUN+Sema3A- and Sema3A-treated tumors, compared with controls. (**C**) Bar graph shows the percent of FITC-lectin-perfused blood vessels (green) on total area. SUN+Sema3A treatment enhanced vessel perfusion by 76% compared to SUN-treated insulinomas. Sema3A improved vessel perfusion by 27% compared to controls. (**D**) Bar graph shows the percent of FITC-dextran extravasation (green) on total area. SUN+Sema3A treatment reduced vessel permeability by 86%

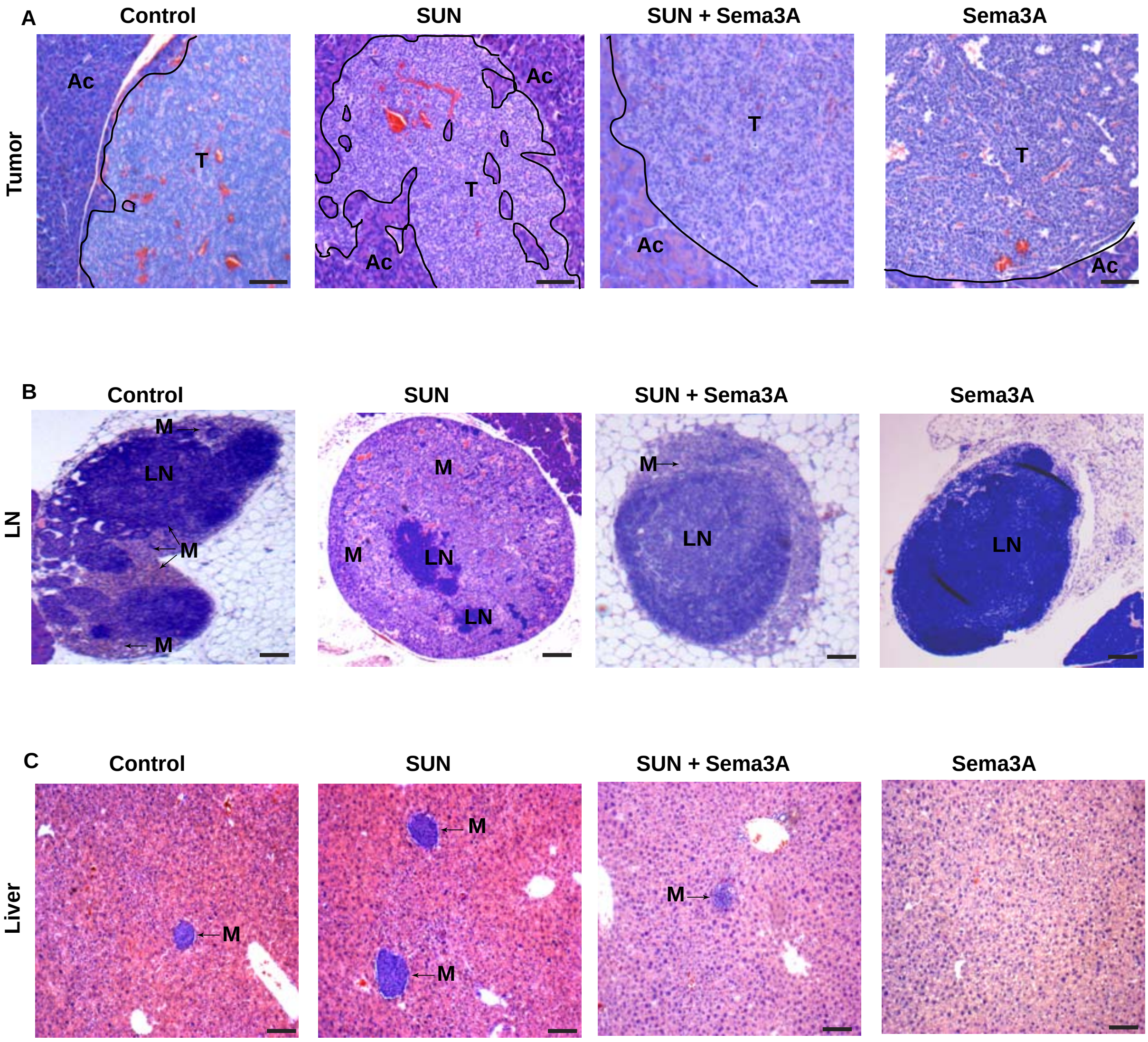
compared with SUN-treated insulinomas. Sema3A decreased dextran extravasation by 83% compared with controls. Values are mean ± SD of 5 fields per tumor per mouse from a total of 6 mice per treatment group. Unpaired Mann-Whitney U-test (*p<0.05, **p<0.01). Scale bars: 50 μm.

Supplemental Figure 7. Sema3A halts metastasis dissemination caused by DC101 treatment in RIP-Tag2 mice. T-Ag immunostaining was used to determine the degree of LN and liver metastasis formation. Bar graphs show the calculation of the number (**A**) and volume (**B**) of liver metastasis. DC101+AAV8-Sema3A (DC101+Sema3A) treatment diminished the number and volume of liver metastasis by 88% and by 86%, compared to DC101 treatment. Sema3A reduced the number and volume of liver metastasis by 80% and by 85%, compared with controls, respectively. (C) Bar graph indicates the measurement of the volume of LN metastasis. DC101+Sema3A treatment lessened LN volume by 94%, compared with DC101 treatment. Sema3A decreased LN volume by 79% compared with controls. Values are mean ± SD (n=15 mice per treatment group). Unpaired Mann-Whitney U-test (*p<0.05, **p<0.01, ***p<0.001).

Supplemental Figure 8. Sema3A alone or combined with DC101 improves tumor tissue perfusion and decreases blood vessel permeability in RIP-Tag2 mice. (**A-D**) Vessel perfusion and permeability were assessed by means of confocal microscopy analysis and by FITC-lectin (**A**) and FITC-70KDa dextran extravasation (**B**) immunostaining, respectively, in DC101-, DC101+Sema3A- and Sema3A-treated tumors, compared with controls. (**C**) Bar graph shows the percent of FITC-lectin-perfused blood vessels (green) on total area. DC101+Sema3A treatment enhanced vessel perfusion by 60% compared with DC101-treated insulinomas. Sema3A improved vessel perfusion by 38% compared with controls. (**D**) Bar graph shows the percent of FITC-dextran extravasation (green) on total area. DC101+Sema3A treatment reduced vessel permeability by 95% compared with DC101-treated insulinomas. Sema3A decreased dextran extravasation by 94%

compared with controls. Values are mean ± SD of 5 fields per tumor per mouse from a total of 8 mice per treatment group. Unpaired Mann-Whitney U-test (**$p<0.01$). Scale bars: 50 μm.

Supplemental Figure 1



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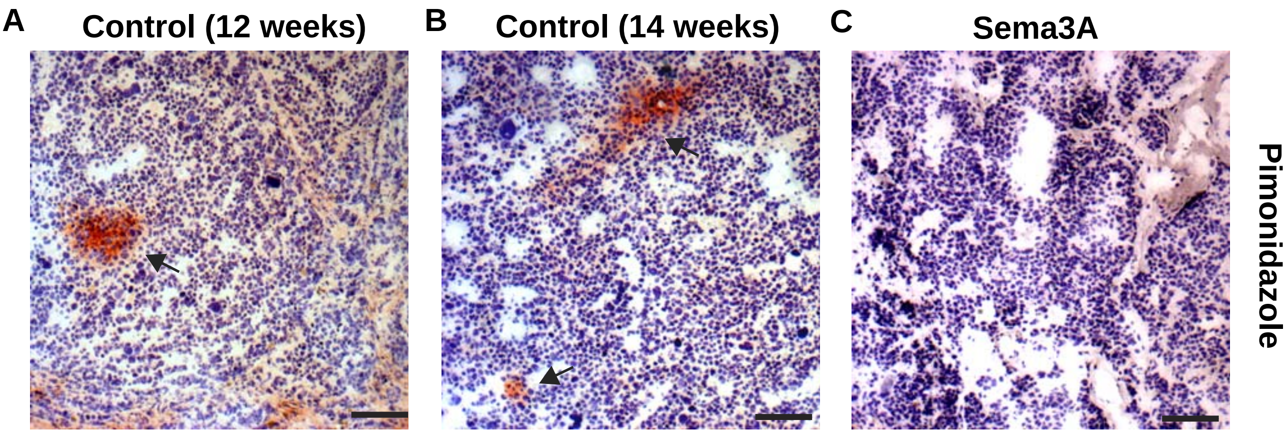

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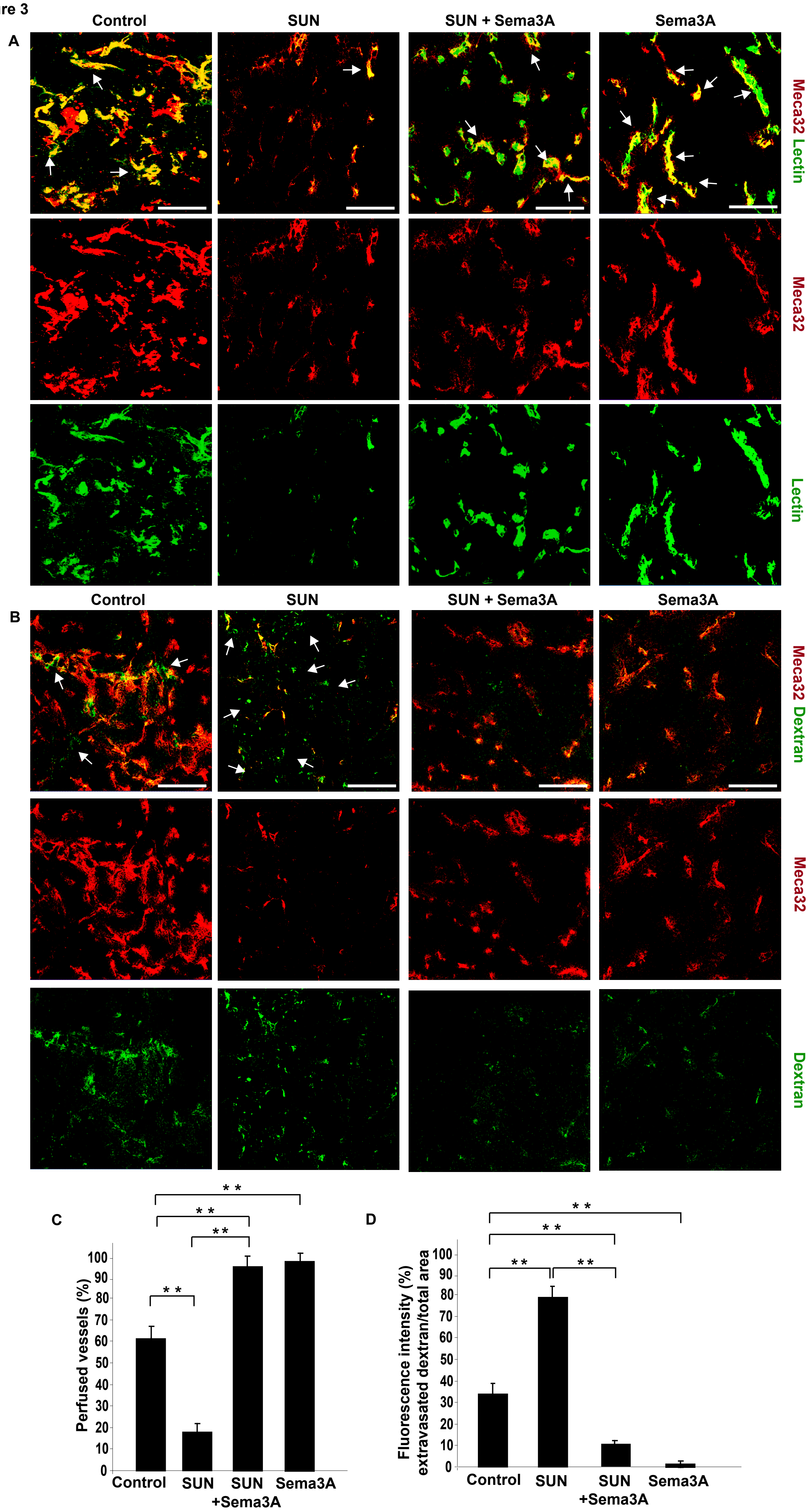

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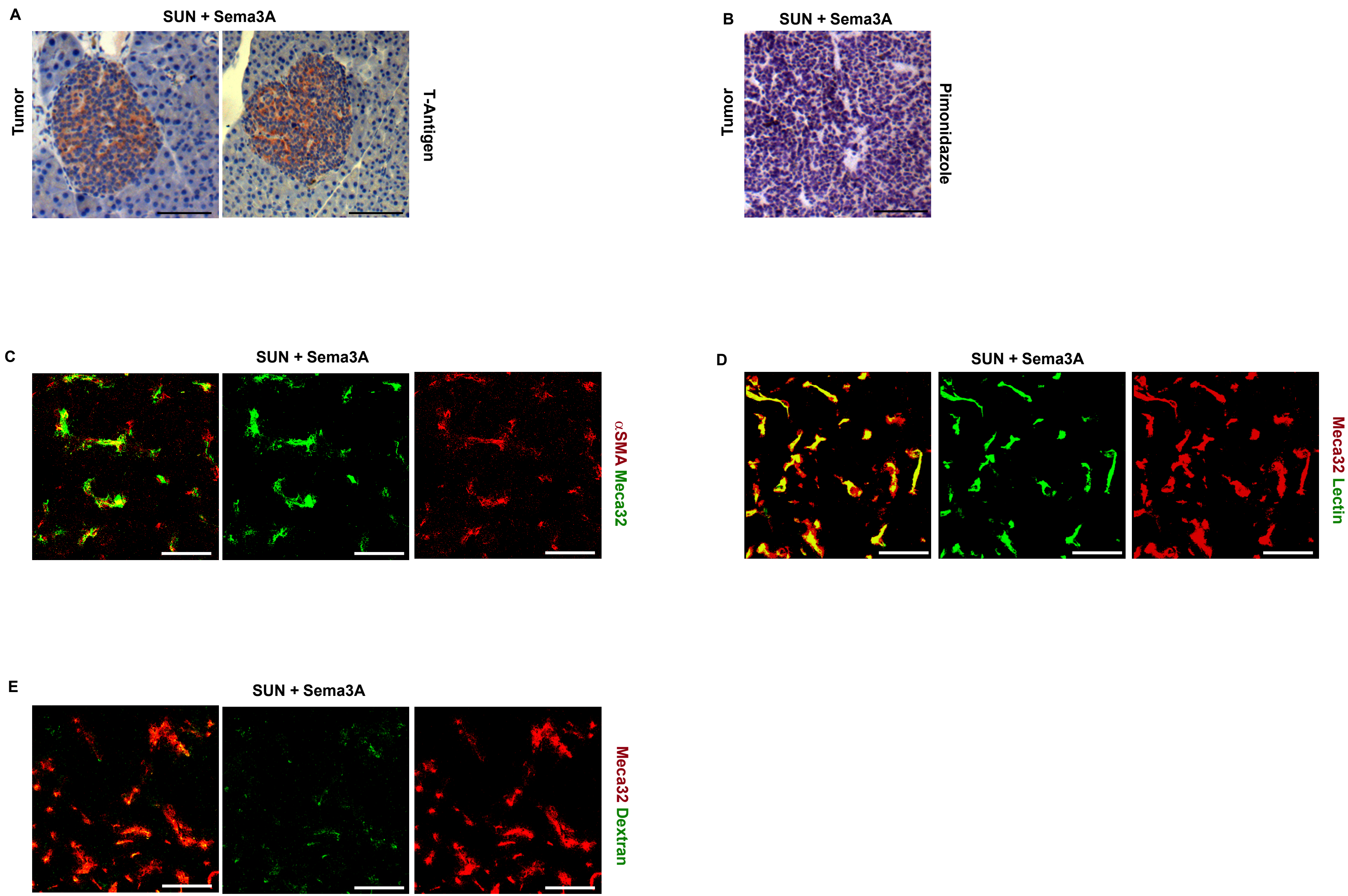

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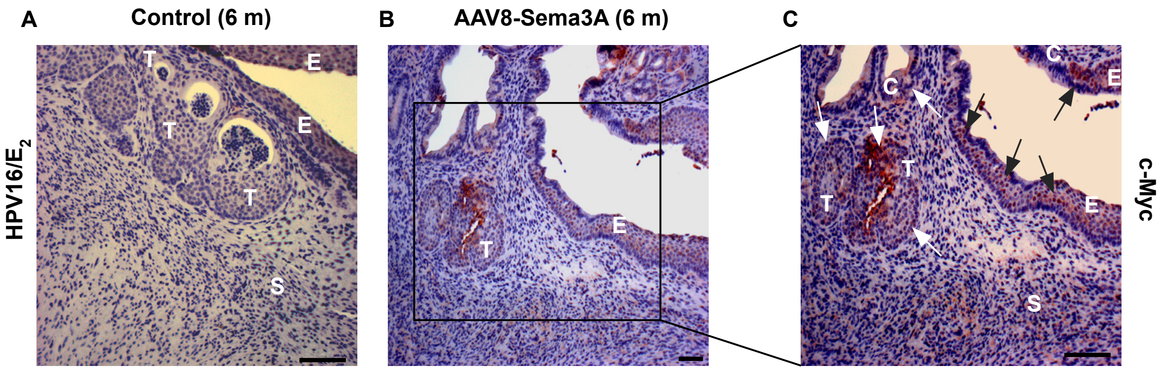

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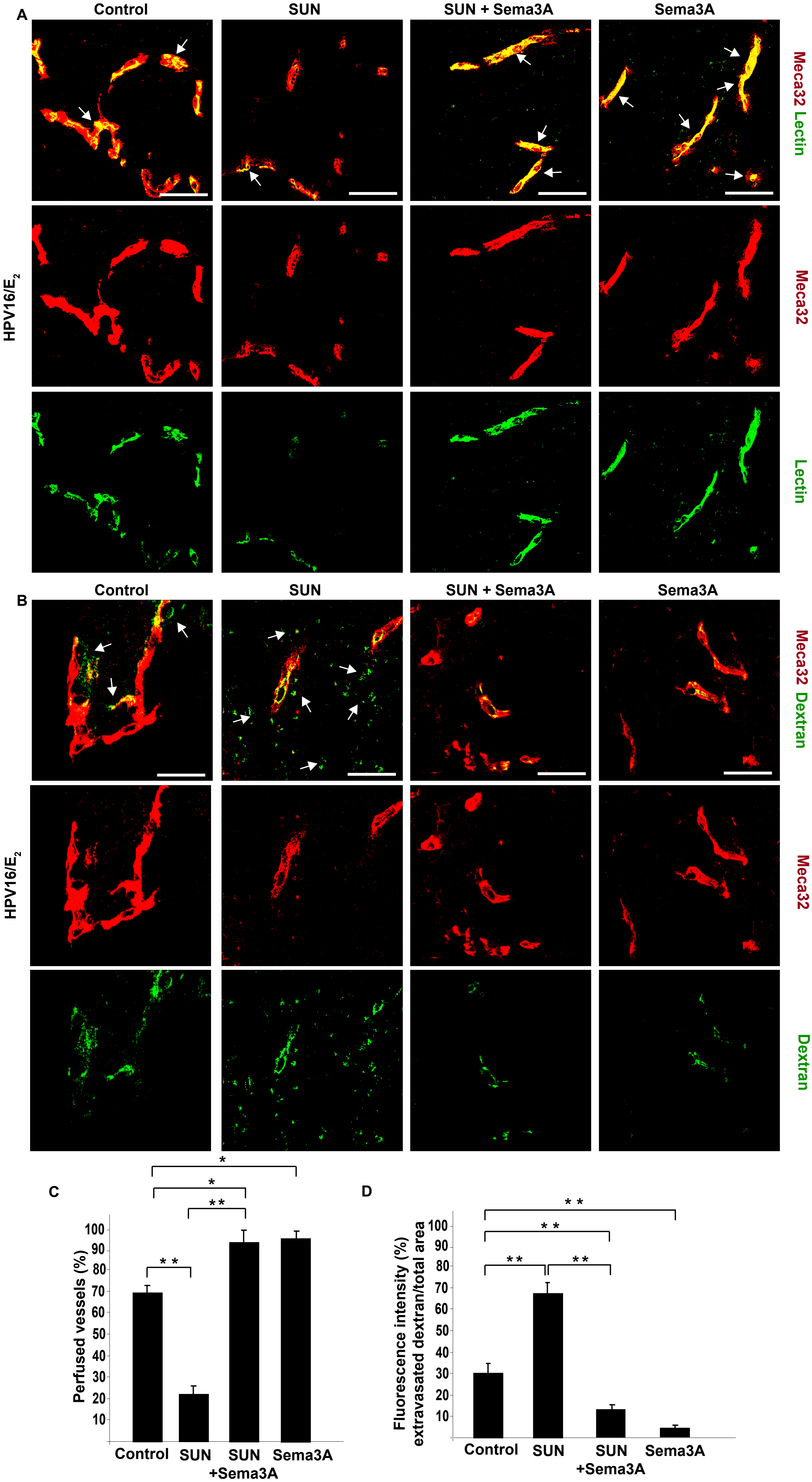
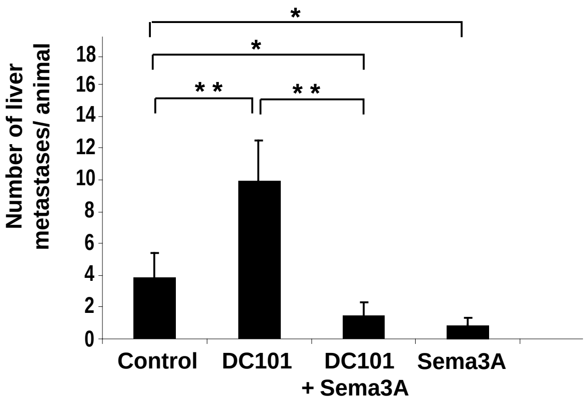


Supplemental Figure 7

A



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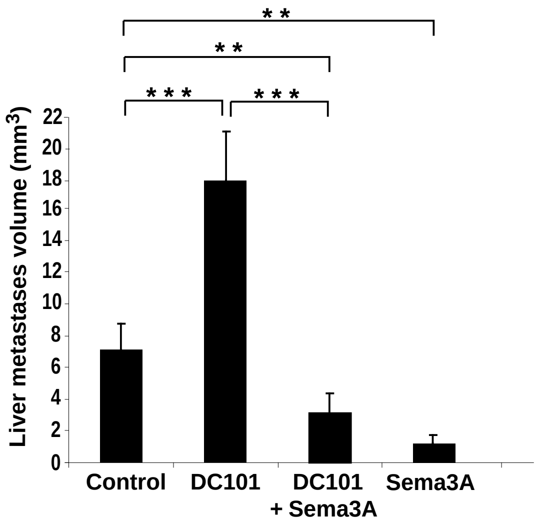

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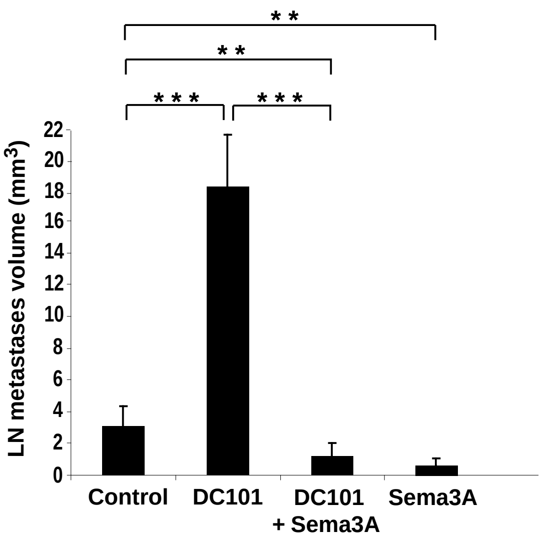

Supplemental Figure 8