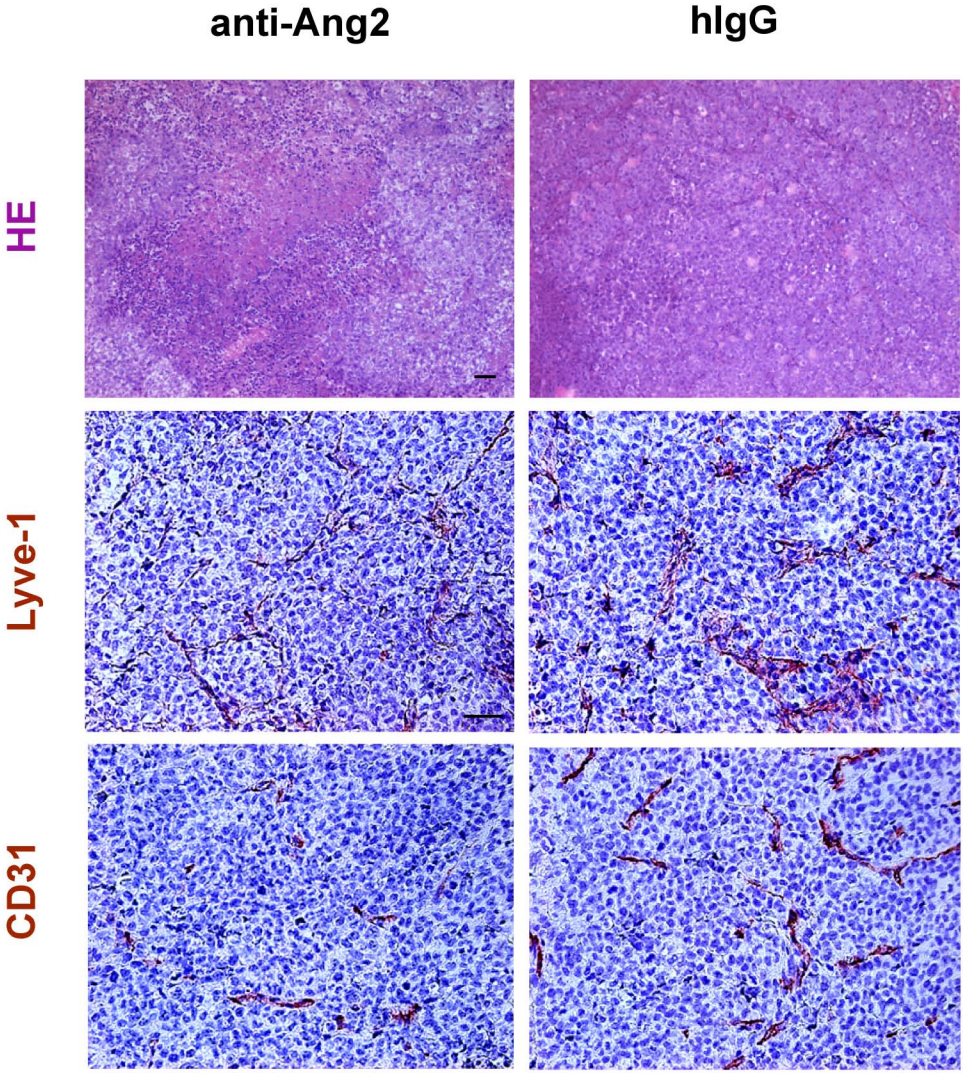
Supplementary Fig. 1. Inhibition of tumor angiogenesis and lymphangiogenesis by Ang2 blocking antibody. Representative images of hematoxylin-eosin (HE) stained LNM35 tumor sections from mice treated with anti-Ang2 antibody or control. Immunohistochemical images of CD31 and LYVE-1 stained tumor sections with hematoxylin counterstaining. Scale bar, 120 μm. anti-Ang2 = Angiopoietin-2 blocking antibody, hIgG = human immunoglobulin G; CD31 = cluster of differentiation 31; Lyve-1 = lymphatic vessel endothelial hyaluronan receptor-1.



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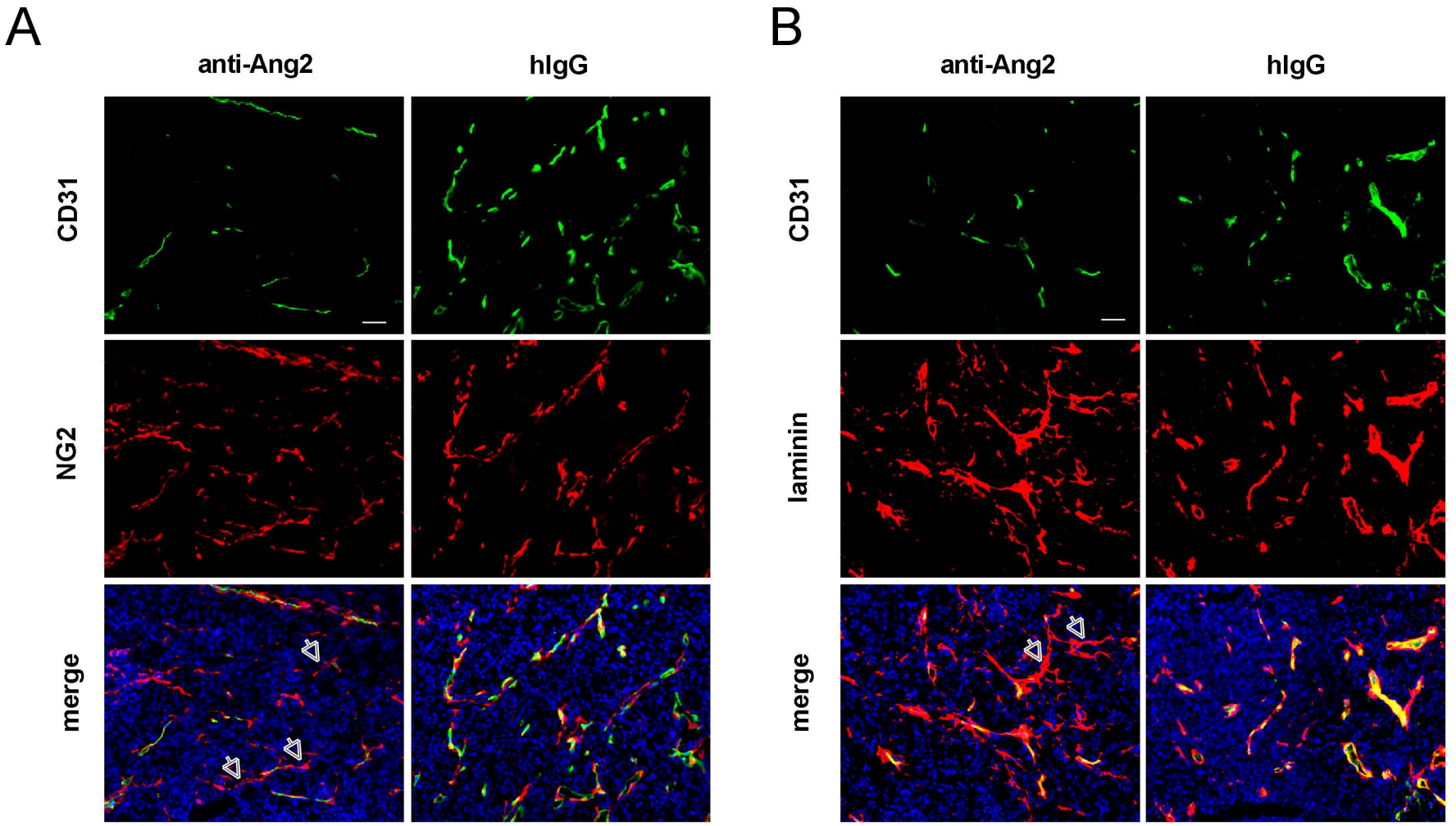
Supplementary Fig. 2. Analysis of the blood capillary network in tumors treated with the Ang2 blocking antibody. **A.** Representative images of LNM35 tumor sections from nu/nu mice stained for CD31+NG2. Arrows indicate pericytes extending from CD31 positive vascular endothelium in the anti-Ang2 treated tumors. **B.** Immunostaining of LNM35 tumors for CD31+laminin. Arrows indicate laminin positive structures coating the blood vessels. Note that some of the endothelium is greatly attenuated inside of the basement membrane sleeves. Scale bars, 120 μm. anti-Ang2 = Angiopoietin-2 blocking antibody; hIgG = human immunoglobulin G; CD31 = cluster of differentiation 31; NG2 = neural/glial antigen 2.



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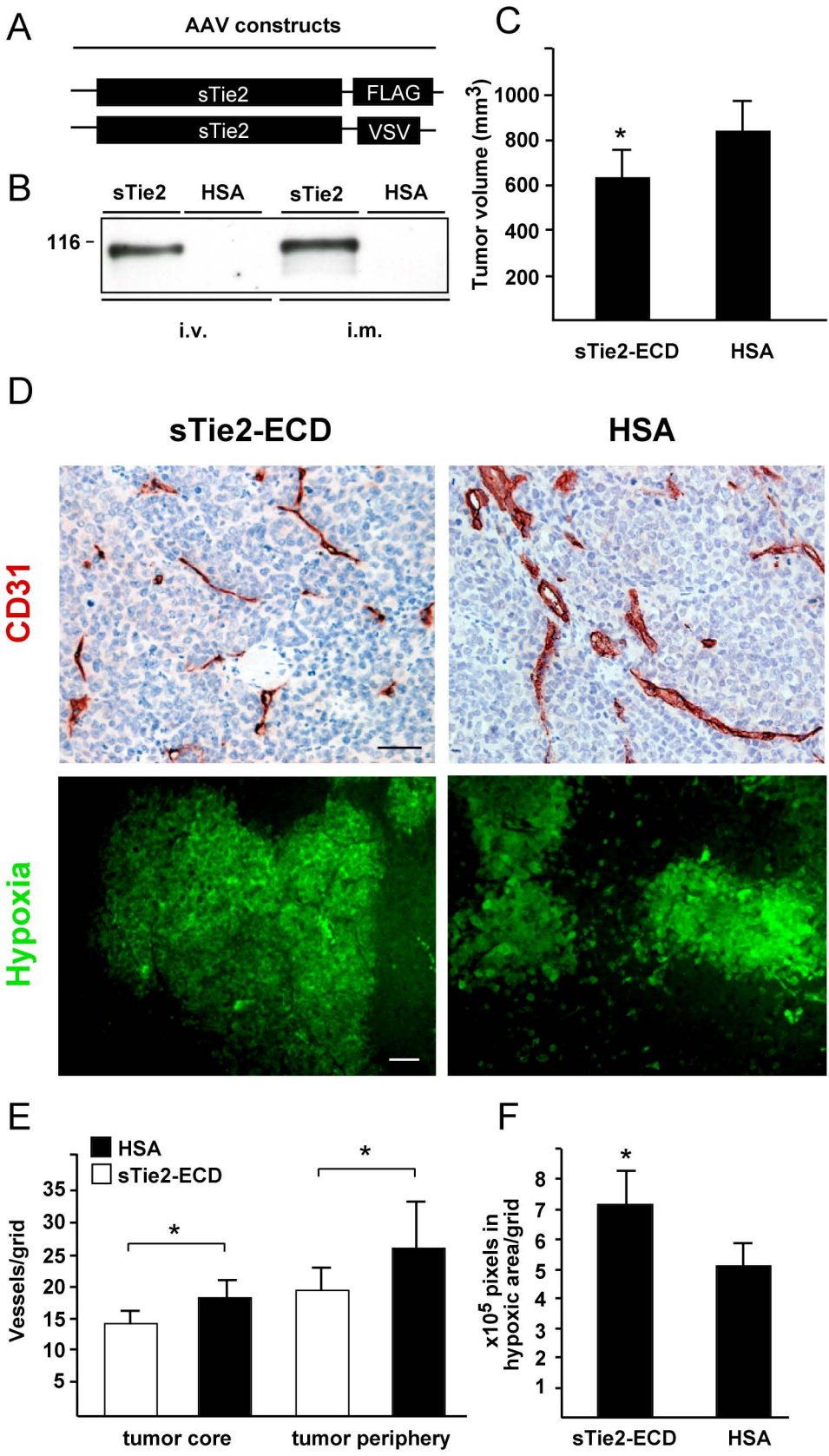
Supplementary Fig. 3. Effect of soluble Tie2-ECD delivered via AAV on primary tumor growth and angiogenesis. **A.** Schematic structures of the soluble Tie2 constructs. **B.** Western blot analysis of sTie2-ECD in serum after systemic or intramuscular injection of AAV-sTie2-ECD or AAV-HSA. **C.** Comparison of LNM35 primary tumor growth (tumor volume: sTie2-ECD vs HSA, 632 mm^3 vs 819 mm^3, n = 14 in both groups, difference = 188 mm^3, 95 % CI = 6 to 369 mm^3, *P* = .043). **D.** Representative images of tumor sections stained for CD31 (upper panel) or pimonidazole adducts (lower panel). **E.** Quantification of blood vessel density within the tumor core and at the periphery of the tumors (intratumoral CD31+ positive vessels/grid: sTie2-ECD [n = 5] vs HSA [n = 4], 14.65 vs 18.67, difference = 4.02, 95 % CI = 1.21 to 6.82, *P* = .012, peritumoral vessels: sTie2-ECD vs HAS, 19.63 vs 26.17, difference = 6.54, 95 % CI = 0.55 to 12.52, *P* = .036. **F.** Quantification of tumor hypoxia, pixels/grid: sTie2-ECD vs HSA, 7.16 $x10^5$ vs 5.07 $x10^5$, difference = 2.09 $x10^5$, 95 % CI = 0.96 to 3.20 $x10^5$, *P* = .003. Scale bar, 100 μm. Error bars = 95 % CI. **P* < .05. Student *t* test. All statistical tests were two-sided. CI = confidence interval. AAV = adeno-associated virus; sTie2/sTie2-ECD = soluble Tie2 extracellular domain, HSA = human serum albumin, i.v. = intravenous, i.m. intramuscular, CD31 = cluster of differentiation 31.



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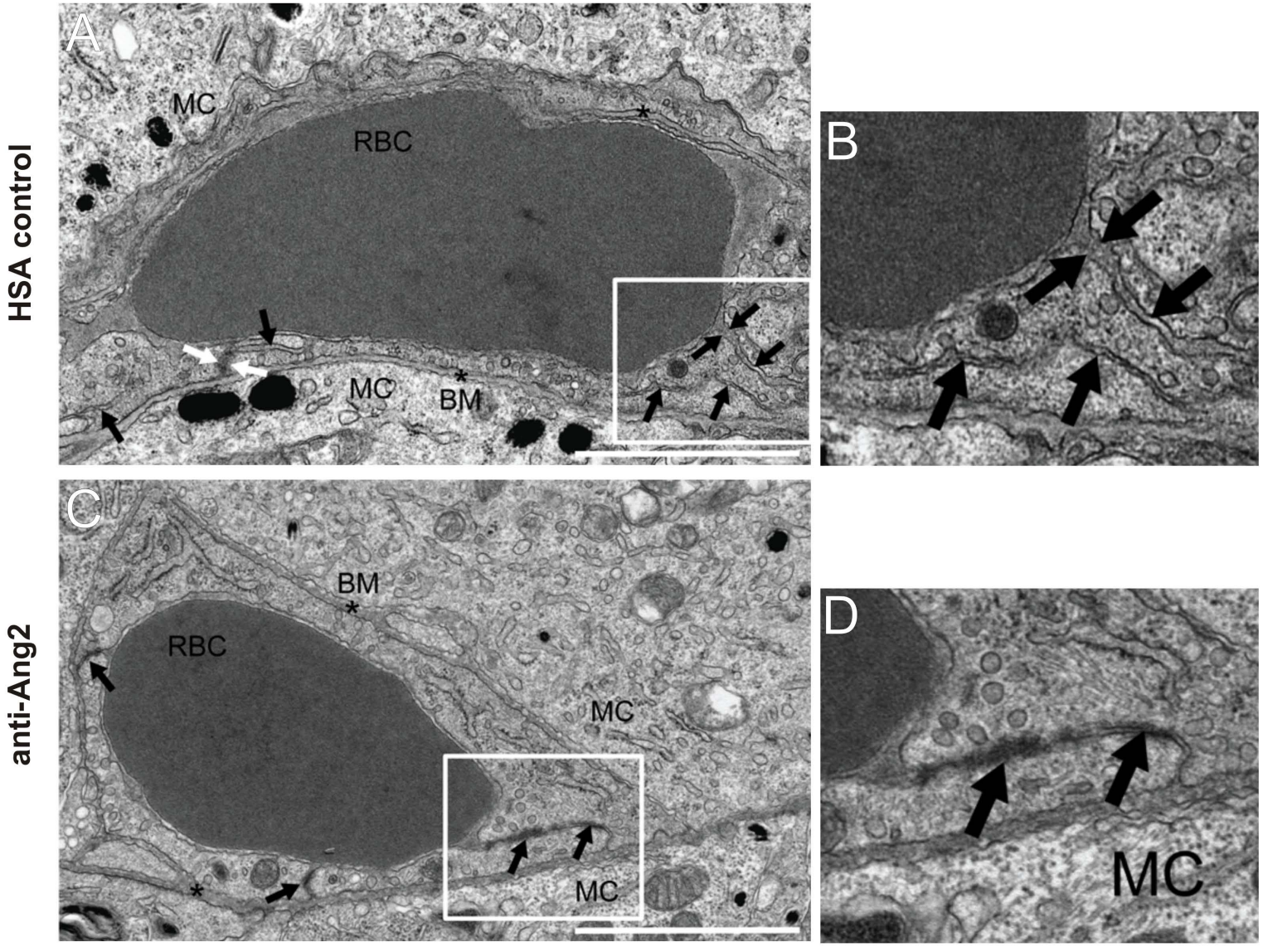
Supplementary Fig. 4. Effect of Ang2 blocking antibody on endothelial cell-cell junctions in lung metastases. Transmission electron micrographs of Ang2 blocking antibody and HSA control treated capillaries of B16F10 melanoma cell (MC) metastases in immunocompromized mice. Note that the Ang2 blocking antibody treatment has a normalizing effect; the junctional complexes between endothelial cells are more prominent in the anti-Ang2 antibody treated lungs (black arrows; white arrows point to a "normal" EC-EC junction). EC = endothelial cell, RBC = red blood cell, anti-Ang2 = Angiopoietin-2 blocking antibody, HSA = human serum albumin. Scale bar 2 μm.



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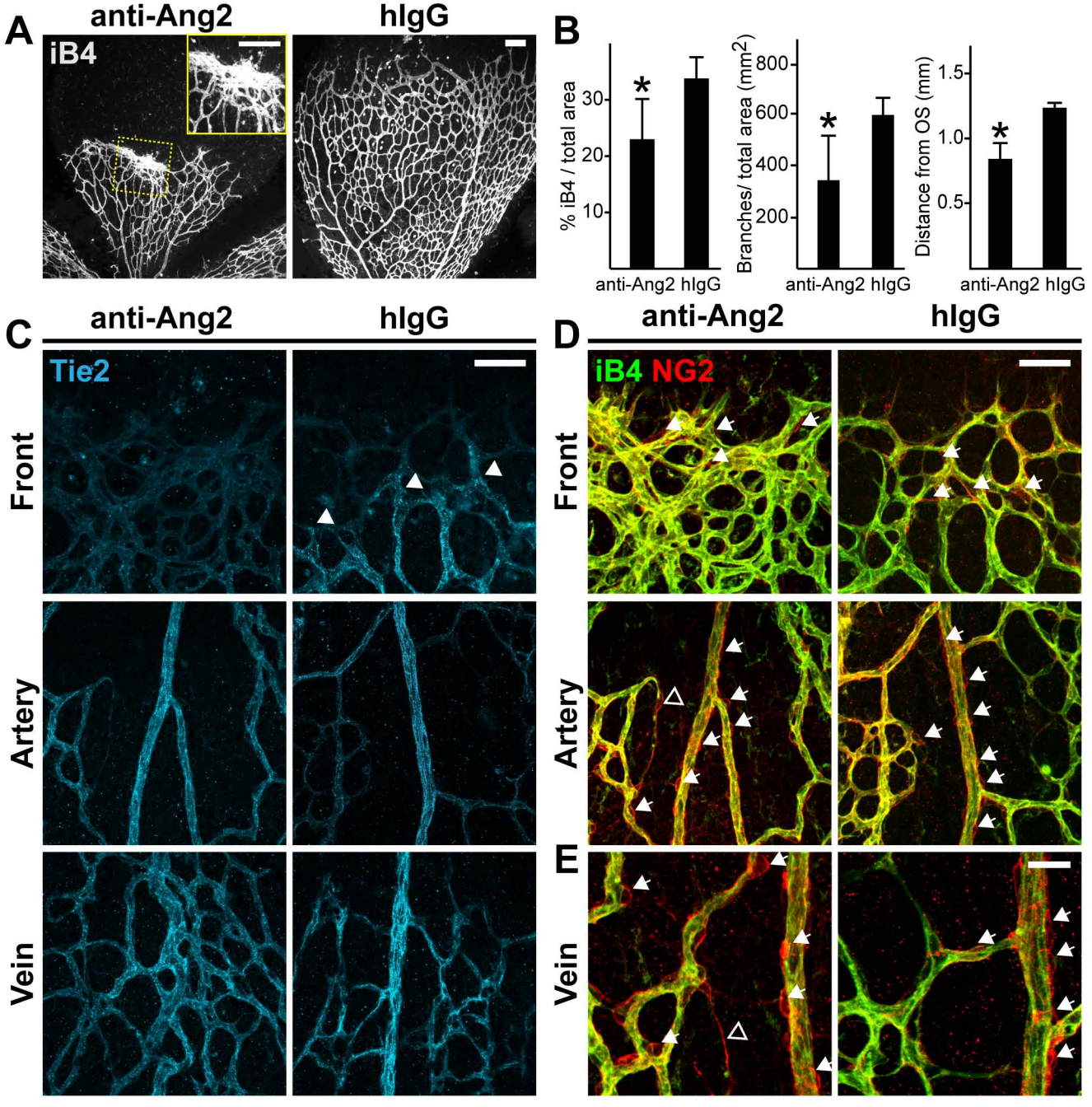
Supplementary Fig. 5. Analysis of the effects of the Ang2 blocking antibodies in the postnatal mouse retina. **A)** Isolectin B4 staining of P5 retinas of NMRI pups treated with the Ang2 blocking antibodies or hIgG during P0-P4. Note retinal tuft-like structures after the Ang2 blocking treatment. **B)** Quantification of the retinas shown in A: iB4 positive surface area normalized to total area, number of vessel branching points, and extent of vascular plexus migration from the optic stalk (OS), iB4/total area: anti-Ang2 group [n = 3] vs hIgG group [n = 4], 23.0 % vs 33.8 %, difference = 10.8 %, 95 % CI = 5.7 % to 15.8 %, *P* = .003; branches/total area: anti-Ang2 group vs hIgG group, 346 mm^2 vs 602 mm^2, difference = 256 mm^2, 95 % CI = 147 mm^2 to 364, mm^2 *P* = .002; distance from OS, anti-Ang2 vs hIgG, 0.84 mm vs 1.23 mm, difference = 0.39 mm, 95 % CI = 0.32 mm to 0.47 mm, *P* < .001. **C)** Decreased Tie2 expression in the retinal front (arrows), after treatment with the Ang2 blocking antibodies. **D, E)** Staining of pericytes (NG2 (red), indicated with filled arrows) and blood vessels (iB4 (green)). Empty arrows indicate pericytes, which are not associated with vessels. *P<.05. Student *t* test. All statistical tests were two-sided. CI = confidence interval; anti-Ang2 = Angiopoietin-2 blocking antibody, hIgG = human immunoglobulin G, iB4 = isolectin B4. Scale bar in **A**, 100 μm, in **C** and **D**, 50 μm and in **E**, 20 μm.



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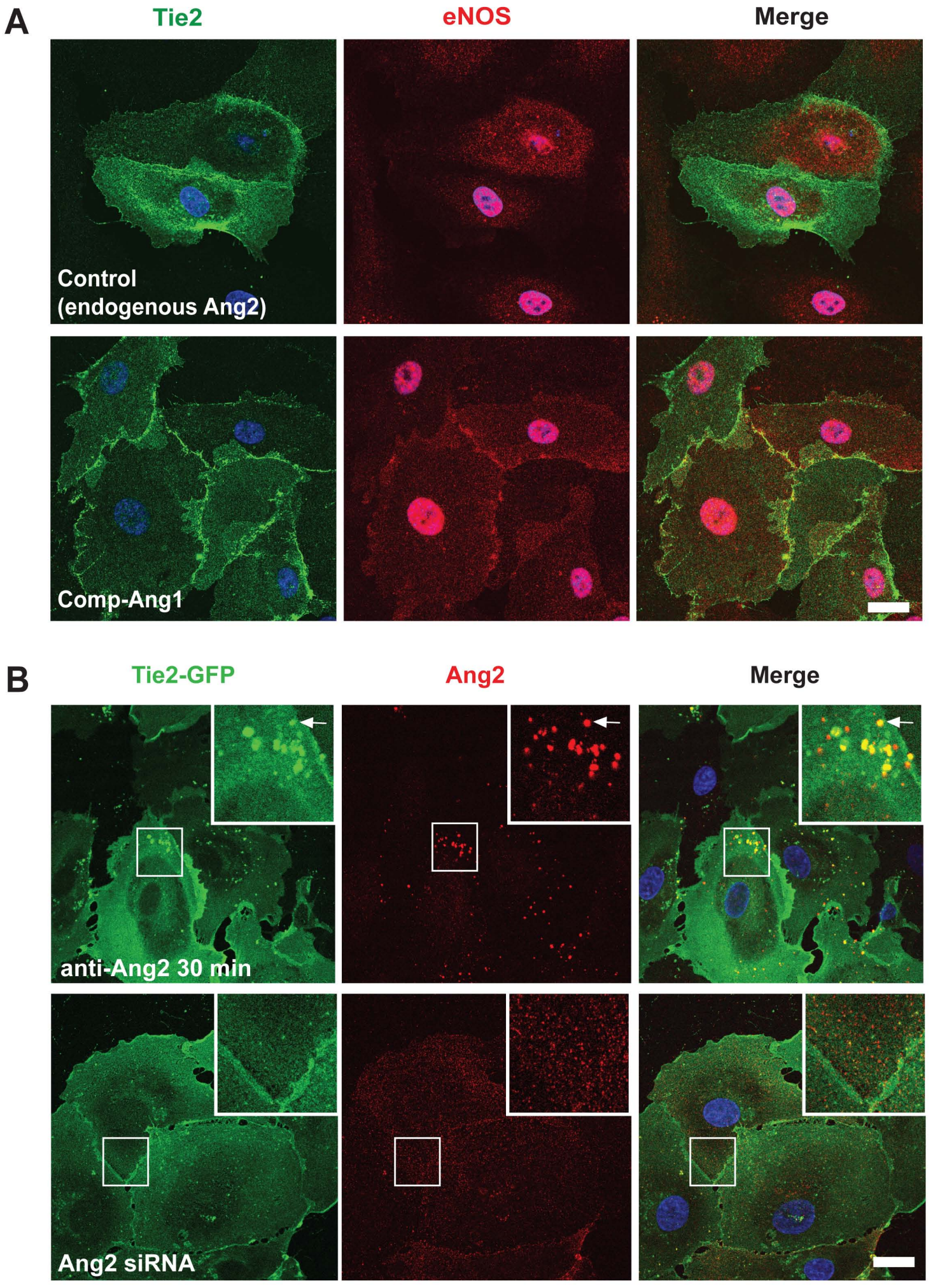
Supplementary Fig. 6. Effects of Ang1, autocrine Ang2, anti-Ang2 antibody, and Ang2 siRNA on eNOS phosphorylation, eNOS activation, and internalization of Ang2-Tie2 receptor complexes. **A.** Representative images of phospho-eNOS staining of Tie2-GFP retrovirus transfected BECs treated with COMP-Ang1 or control to monitor autocrine Ang2. Note that Ang1 induces eNOS activation, while Ang2 does not influence eNOS phosphorylation. **B.** Tie2-GFP retrovirus transfected BECs were treated with Ang2 or control siRNA, and the control or Ang2 blocking antibodies, respectively, were applied onto cells for 1 hour and then fixed, permeabilized and stained for Ang2. Note that the anti-Ang2 antibody induces internalization of Ang2 and Tie2 in intracellular vesicles, while vesicles are not detected in Ang2 siRNA transfected cells. Note also that in cells transfected with Ang2 siRNA or treated with anti-Ang2 no Tie2 complexes are present in cell-cell junctions. Nuclear DAPI stain. Confocal microscopy images. Scale bar, 20 μm. COMP-Ang1 = cartilage oligomeric matrix protein -Angiopoietin-1, eNOS = endothelial nitric oxide synthase, anti-Ang2 = Angiopoietin-2 blocking antibody, Ang2 = Ang2 detecting antibody, BECs = Blood endothelial cells.



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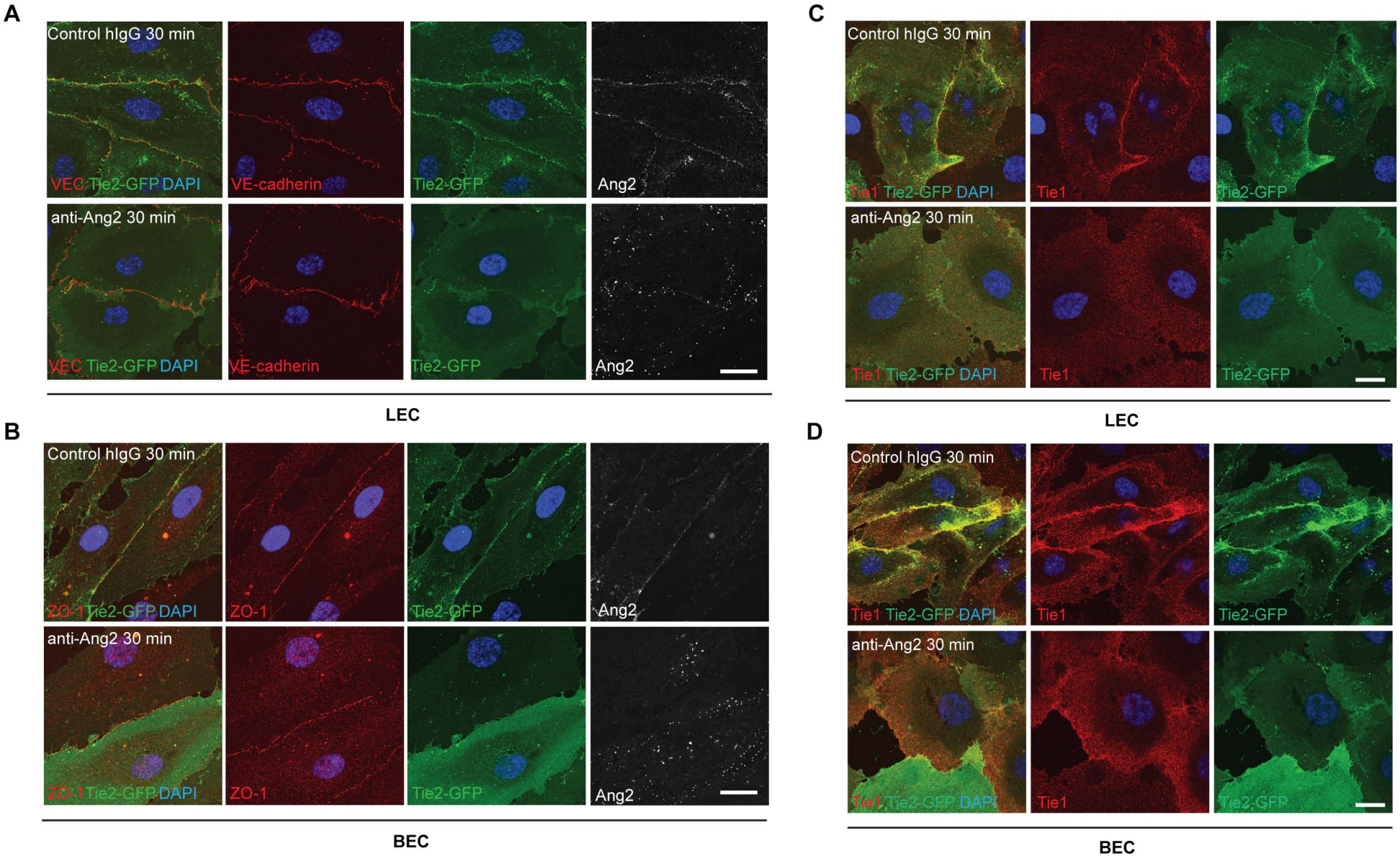
Supplementary Fig. 7. Effect of anti-Ang2 antibody on Ang2-induced translocation of Tie1 and Tie2 to cell-cell junctions. LECs (**A, C**) and BECs (**B, D**) were transfected with Tie2-GFP, and treated for 30 minutes with anti-Ang2 antibody or hIgG as a control. The cells were fixed, permeabilized and stained for VE-Cadherin and Ang2 (**A**), ZO-1 and Ang2 (**B**) or Tie1 (**C, D**). Ang2 blocking antibody inhibits Ang2-induced translocation of Tie1 and Tie2 receptors to cell-cell junctions. Nuclear DAPI stain. Confocal microscopy images. Scale bar, 20 μm. anti-Ang2 = Angiopoietin-2 blocking antibody; LECs = lymphatic endothelial cells; BECs = blood endothelial cells; GFP = green fluorescent protein; VE-cadherin = vascular endothelial cadherin; DAPI = 4',6-diamidino-2-phenylindole.



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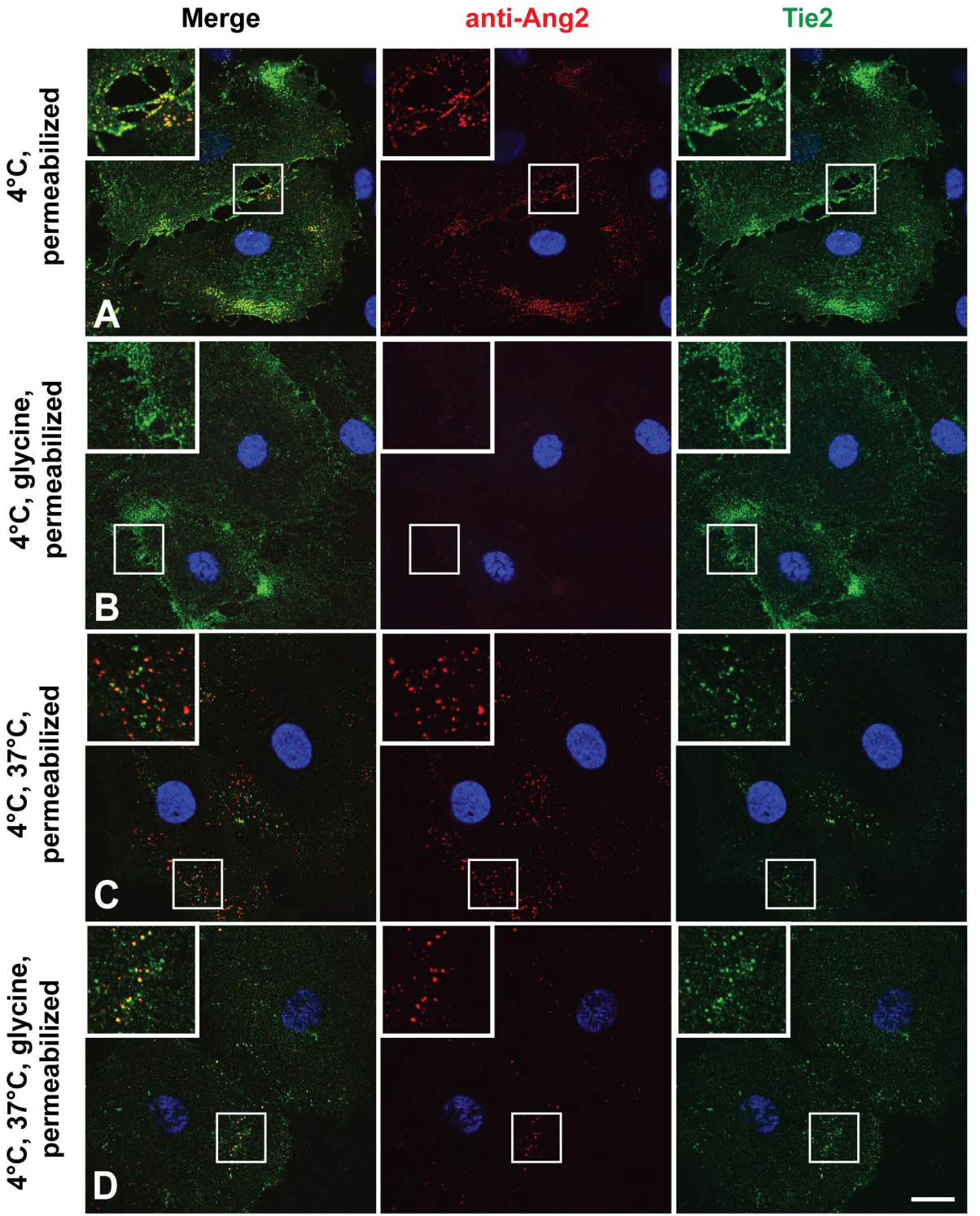
Supplementary Fig. 8. Analysis of internalization of cell surface bound anti-Ang2 antibody in LECs. **A-D.** Representative images of analysis of internalization of cell surface bound anti-Ang2 antibody after depletion of the cell surface with low pH buffer. Anti-Ang2 antibodies were incubated with cells on ice, and fixed **(A)**, or subjected to acid wash to remove cell surface bound antibodies before fixing **(B)**. (**C, D)** After incubation on ice, the cells were transferred to 37^{o}C for 30 min, and fixed **(C)**, or subjected to acid wash before fixing **(D)**. The anti-Ang2 antibodies were detected after permeabilization of the cells using fluorochrome-conjugated anti-human secondary antibodies (red) and Tie2 using anti-Tie2 antibodies (green). Note that acid wash completely removes antibody in **B**, but internalized antibodies are protected in **D**. Confocal microscopy images. Scale bar, 20 μm. anti-Ang2 = Angiopoietin-2 blocking antibody. LECs = lymphatic endothelial cells.



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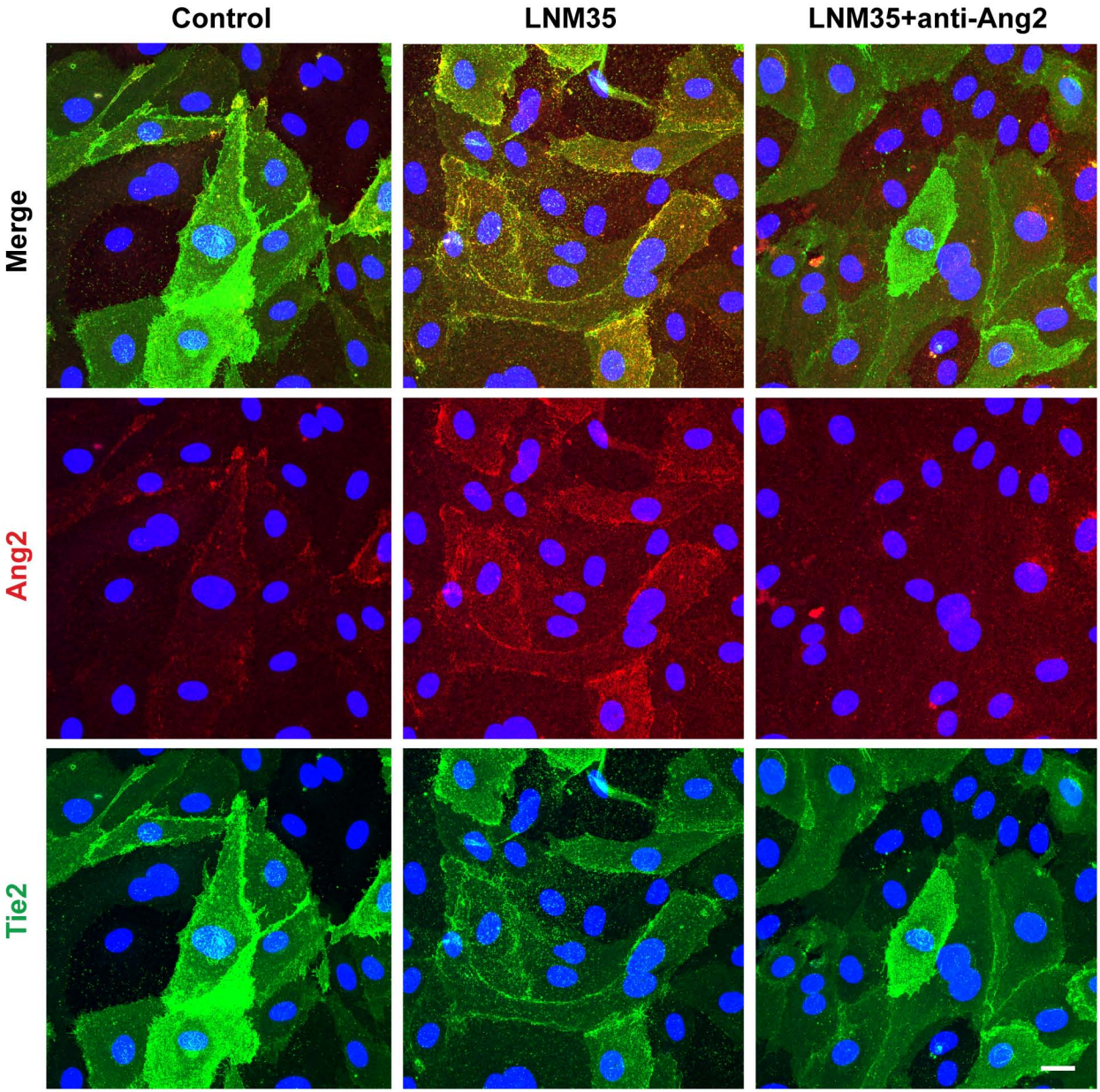
Supplementary Fig. 9. Effect of Ang2 blocking antibodies on tumor cell-induced Ang2-Tie2 complex formation in endothelial cells. Tie2-GFP expressing BECs were cultured on Transwell filters with or without LNM35 cells in the bottom well in the presence or absence of the Ang2 blocking antibodies. The BECs were fixed, permeabilized and stained for Ang2. Note increased Ang2 localization at cell-cell contacts in BECs cultured in the presence of the LNM35 cells. Ang2 cell surface binding, but not Ang2 intracellular fluorescence is blocked by Ang2 blocking antibodies, as shown by the Ang2 staining and uniform Tie2 signal on the BECs. Stacked confocal images were created using the LSM software (Zeiss LSM 510 Meta, Zeiss LSM 5 Duo [Carl Zeiss AG], Olympus FluorView FV1000 (Olympus, Tokyo, Japan). Scale bar, 20 μm. anti-Ang2 = Angiopoietin-2 blocking antibody, Ang2 = Angiopoietin-2 detecting antibody; BECs = blood endothelial cells.



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