Supplementary data

Endothelial cell tube formation

Each well of a 24-well plate were coated with 250 μL of Matrigel® Basement Membrane Matrix (Corning, Bedford, MA, USA) without trapping air bubbles and settled at 37°C for at least 30 min as allowance for gelation of Matrigel® Matrix. MG-63 cells and HUVECs were seeded at 5.0×10^4 cells·cm^{-2} in each well of a 24-well plate coated with Matrigel® Basement Membrane Matrix and incubated at 37°C and 33°C for 6 h. At the culture time, the cells were observed under phase-contrast microscope (CKX53-11BFK and DP21-SAL, Olympus, Tokyo, Japan). The tube formation images were quantified using Angiogenesis Analyzer for ImageJ software [1].

Figure S1 Comparison of tube formation in co-culture cells

MG-63 cells and HUVECs were respectively seeded initial density of 1.0×10^4 cells·cm^{-2} and 4.0×10^4 cells·cm^{-2} in 24-well plate coated with Matrigel® Basement Membrane Matrix and cultured at 37°C and 33°C for 6 hours. The cells were (A) viewed with a phase-contrast microscope at 10× magnification (scale bar: 200 μm) and (B) assessed using Angiogenesis Analyzer for ImageJ software. Data were determined from three replicate samples and are shown as the mean ± SD. $^{**}p < 0.01$, compared with 37°C.

Immunofluorescent staining for connexin 43 expression

Co-culture cells were washed with PBS and fixed with 4% paraformaldehyde/PBS for 15 min. The cells were then permeabilized with acetone/methanol (–20°C) at room temperature for 2 min. After rinsing with PBS twice, the cells were blocked with 3% BSA/PBS at room temperature for 1 h and then incubated with monoclonal mouse anti-human CD31 antibody (Dako, Glostrup Denmark) and polyclonal rabbit anti-connexin 43 (Cx43) antibody (Abcam, Cambridge, UK) diluted in PBS at 4°C overnight. The cells were stained with Alexa Fluor™ 594-labled goat anti-mouse IgG_1 (Invitrogen, Carlsbad, CA, USA) for CD31, Alexa Fluor™ 488-labled goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for Cx43, and DAPI (Dojindo, Kumamoto, Japan) for nuclei diluted in PBS. The cells were again washed with PBS and then examined with fluorescence phase-contrast microscope (BZ X-710, Keyence, Osaka, Japan).

Figure S2 Immunofluorescent staining for Cx43 expression in co-culture cells

MG-63 cells and HUVECs were respectively seeded initial density of 2.0×10^4 cells·cm^{-2} and 8.0×10^4 cells·cm^{-2} in 24-well plate and precultured at 37°C for 24 hours. Cells were cultured at 37°C and 33°C for 7, 14, and 21 days. At the culture time, the cells were fixed and stained (A) with anti-Cx43 for Cx43 (green), anti-human CD31 for CD31 (red), and DAPI for nucleic (blue). They were viewed with a fluorescence phase-contrast microscope 20× magnifications (scale bar: 100 μm).

Abbreviations: HUVECs, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Cx43, connexin 43; DAPI, 4',6-diamino-2-phenylindole.

Reference

1. Carpentier G (2012) ImageJ contribution: angiogenesis analyzer. ImageJ News.

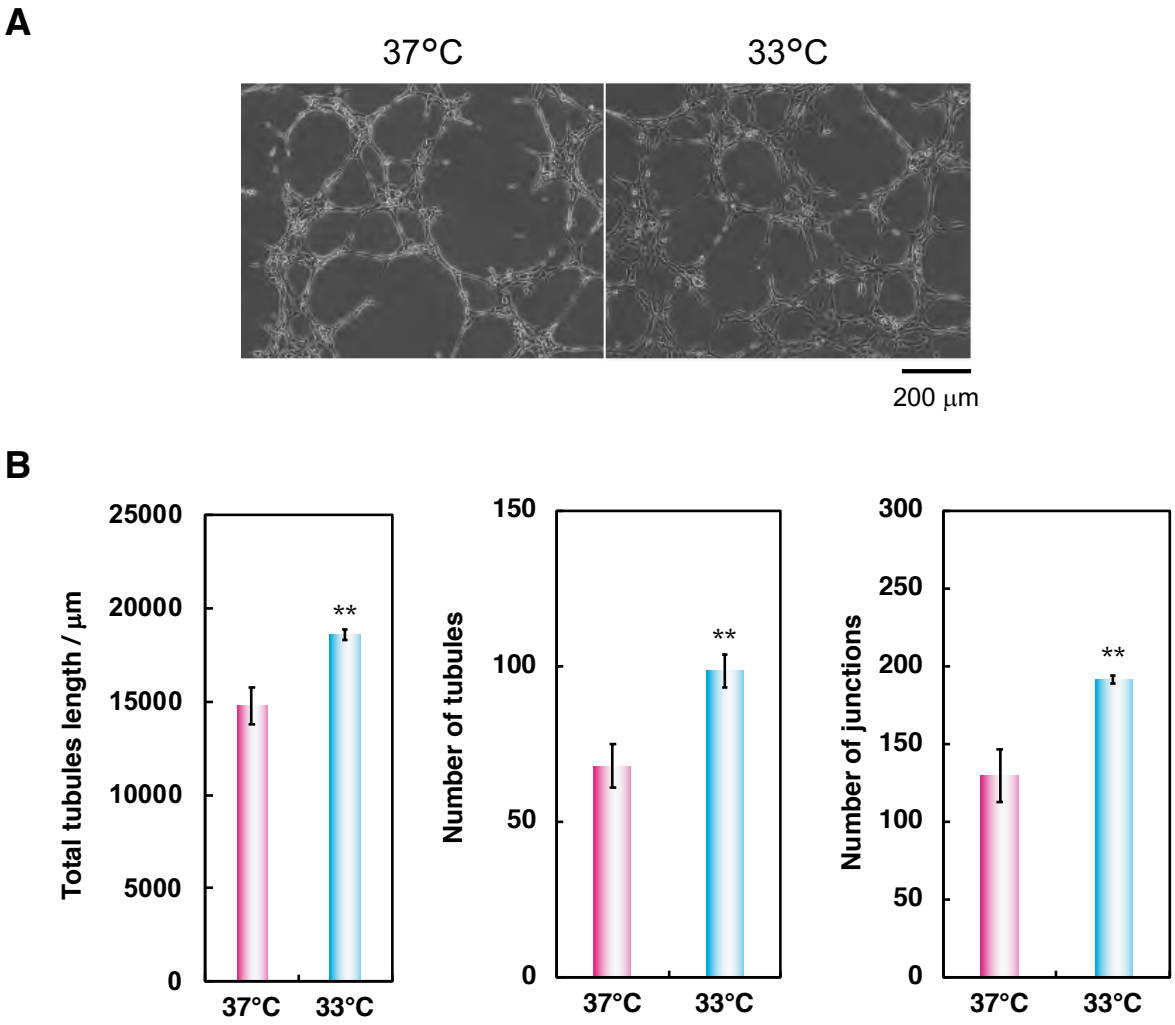

Fig. S1 K. Inomata and M. Honda

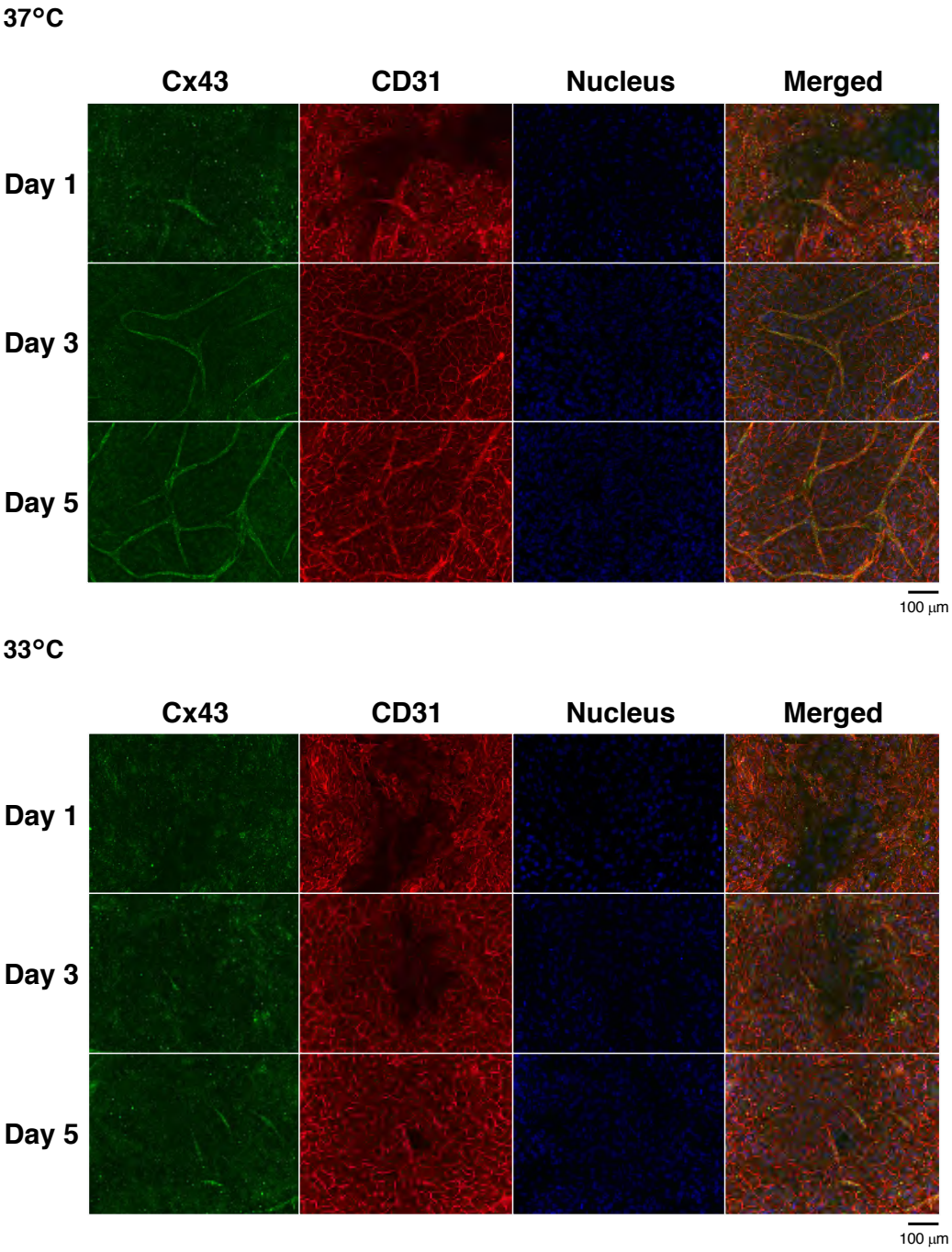

Fig. S2 K. Inomata and M. Honda