

Research article

Repeated sequential administration of pegylated emulsion of SU5416 and liposomal paclitaxel enhances anti-tumor effect in 4T1 breast cancer-bearing mice

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Abstract

To improve vascular normalization strategy for intractable triple-negative breast cancer 4T1, we examined the anti-tumor effects of repeated sequential administration of polyethylene glycol (PEG)-modified emulsion of SU5416 (PE-SU5416), a vascular endothelial growth factor (VEGF) receptor-2 kinase inhibitor, and PEG-modified liposomal paclitaxel (PL-PTX) in mice bearing 4T1 cells. Three sequential administrations (Seq×3) of PE-SU5416 and PL-PTX exhibited significantly higher anti-tumor activity than a single sequential administration (Seq×1). The tumor vasculatures were structurally normalized until after two PE-SU5416 (PE-SU5416×2) or sequential (Seq×2) administrations, while the improvement in vascular function, such as oxygen supply, blood flow, and PEG-liposomal distribution, was evident until after three administrations of PE-SU5416 (PE-SU5416×3) and Seq×3. Although some discrepancies between the structural and functional improvement in tumor vasculatures were observed after PE-SU5416×3 and Seq×3, cancer-associated fibroblasts (CAFs) and collagen levels were significantly reduced after PE-SU5416×2, PE-SU5416×3, Seq×2, and Seq×3, suggesting that a possible decrease in interstitial fluid pressure due to the reduction in CAFs and collagen would have compensated for vascular function. Furthermore, PE-SU5416×2, PE-SU5416×3, Seq×2, and Seq×3 significantly decreased tumor growth

factor- β (TGF- β), an activator of CAFs, in tumor tissues, suggesting that the reduction in TGF- β levels by PE-SU5416 suppresses CAF activation.

Keywords: drug delivery; vascular normalization; breast cancer; liposome; cancer-associated fibroblast.

Abbreviations: α -SMA, α -smooth muscle actin; CAF, cancer-associated fibroblast; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; ECM, extracellular matrix; EPC, Egg yolk phosphatidylcholine; HSPC, hydrogenated soybean phosphatidylcholine; IFP, interstitial fluid pressure; PEG, polyethylene glycol; PEG-DSPE, Distearoyl phosphatidylethanolamine-N-[methoxy poly(ethylene glycol)-2000]; PE-SU5416, PEG-modified O/W emulsion loaded with SU5416; PL-PTX, PEG-modified liposomal paclitaxel; PTX, paclitaxel; Seq, sequential administration; TGF- β , tumor growth factor- β .

1. Introduction

In tumor tissues, angiogenesis is induced for cancer cells to obtain oxygen and nutrients for growth. Endothelial cells forming tumor vessels are disorganized, loosely connected with each other, and form a defective cellular lining of the vessel wall [1,2]. Additionally, pericytes, normally stabilizing blood vessels, abnormally coat endothelial cells [1,2]. Furthermore, the newly formed blood vessels are dilated and tortuous with irregular diameters, and aberrant branching. These abnormal vessel structures lead to aberrant function of tumor vessels such as heterogenous and insufficient blood flow, which is a major obstruction for efficient drug delivery to tumor tissues [3,4].

To overcome this issue, the vascular normalization strategy was proposed to improve vascular abnormalities and thereby drug distribution to tumor tissues utilizing anti-angiogenic agents [5]. These drugs improve vascular abnormalities in a dose- and time-dependent manner, in turn enhancing the delivery of subsequently administered anti-tumor drugs to tumor tissues and their therapeutic efficacy [5–9].

SU5416, a selective vascular endothelial growth factor (VEGF) receptor-2 kinase inhibitor, has been suggested to induce vascular normalization, but its very low aqueous solubility requires frequent intraperitoneal administration as dissolved in DMSO or Cremophor EL, causing several adverse effects and insufficient tumor targeting [10–12].

Previously, we developed an SU5416-loaded polyethylene glycol (PEG)-modified O/W emulsion (PE-SU5416) [13], which made it possible for a single intravenous injection of PE-SU5416 to structurally and functionally improve abnormal tumor vessels and significantly enhance anti-tumor activity by subsequently administering paclitaxel-loaded PEG-modified liposomes (PL-PTX) in mice bearing adenocarcinoma colon-26 [13] and triple-negative breast cancer 4T1 [14]. Furthermore, in the case of 4T1 treatment, we also indicated that PE-SU5416 treatment decreased cancer-associated fibroblasts (CAFs) and collagen, a major component of extracellular matrix (ECM), which led to improved intratumor distribution and anti-tumor efficacy of the subsequently administered PL-PTX [14].

Although vascular normalization therapy for 4T1 tumor-bearing mice with PE-SU5416 and PL-PTX significantly suppressed tumor growth, it was limited to approximately 60% of the control mice [14]. Considering the high malignancy, poor cure rate, lack of targeted therapies, and high probability of metastasis of triple-negative breast cancer [15–17], further improvement in the therapeutic regimen for vascular normalization therapy is required to try to achieve remission of refractory cancers. Several approaches including the changes in doses of PE-SU5416 and/or PL-PTX and in a dosing interval between PE-SU5416 and PL-PTX would be possible, but we have found a good normalization window

[5–9] to improve the drug delivery to tumor cells in our previous study [14].

Therefore, in this study, we decided to take advantage of the normalization window, and examined the effect of repeated sequential administrations of PE-SU5416 and PL-PTX on therapeutic efficacy in mice bearing triple-negative breast cancer 4T1 cells. Three sequential administrations of PE-SU5416 and PL-PTX (Seq×3) successfully suppressed tumor growth to approximately 26% compared to that of the control. Furthermore, the mechanisms involved in the improved therapeutic efficacy were investigated in detail, revealing an improvement in the structural and functional properties of tumor vessels and the tumor microenvironment, which led to improved tumor distribution of PL-PTX.

2. Materials and Methods

2.1. Materials

Chloroform (CHCl₃), Tween 80, tricaprylin, cholesterol, glycerin, and RPMI-1640 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). Egg yolk phosphatidylcholine (EPC) was purchased from Kanto Chemical Co., Inc. (Tokyo). Distearoyl-phosphatidylethanolamine-N-[methoxy poly(ethylene glycol)-2000] (PEG-DSPE) and hydrogenated soybean phosphatidylcholine (HSPC) were obtained from NOF Corporation (Tokyo). Antibiotics (gentamycin, penicillin-streptomycin) and 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). SU5416 and paclitaxel (PTX) were kindly provided by Taiho Pharmaceutical Co., Ltd. (Tokushima, Japan) and Sawai Pharmaceutical Co., Ltd. (Osaka, Japan), respectively. All other chemicals were of analytical grade and use without further purification.

2.2. Preparation of PE-SU5416 and PL-PTX

PE-SU5416 was prepared as described in our previous report [13]. Briefly, tricaprylin, EPC, Tween 80, SU5416 (400:160:120:6.8 weight ratio), and PEG-DSPE (5 mol%) were

117 mixed in CHCl_3 , and evaporated at 60°C in a rotary evaporator under reduced pressure.
118 Then, the resultant oil phase was mixed with 2.25% (w/v) glycerol/phosphate-buffered
119 saline (PBS, pH 7.4) and sonicated using a probe-type sonicator (70 W, Ohtake Works
120 Ltd., Tokyo) for 30 min at 4°C . The loading efficiency of SU5416 in the emulsion (PE-
121 SU5416) was almost 100% and the particle size, polydispersity index (pdi), and zeta
122 potential of the formulation were 134.6 ± 1.2 nm, 0.146 ± 0.016 , and -1.84 ± 0.46 mV,
123 respectively [14].

124 PL-PTX was prepared as reported previously [13,18]. Briefly, CHCl_3 solution
125 containing HSPC, cholesterol, PEG-DSPE, and PTX (90:10:5:8 molar ratio) was
126 evaporated at 75°C in a rotary evaporator and dried under vacuum condition at room
127 temperature (RT) overnight. The resultant lipid film was hydrated with PBS (pH 7.4) by
128 vortex-mixing, and the preparations were extruded sequentially through 200 and 100 nm
129 pore size membrane filters (Millipore, Bedford, MA). For DiI-labeled PEG-liposomes
130 preparation, DiI (10 mol%), a fluorescent marker, was added to the CHCl_3 solution of the
131 lipid mixture as described above. PTX entrapment efficiency in PEG-liposomes was
132 approximately 34%, and the particle size, pdi, and zeta potential of the formulation were
133 96.5 ± 0.5 nm, 0.050 ± 0.003 , and -1.71 ± 0.91 mV, respectively [14].

2.3. Evaluation of anti-tumor activity in 4T1 tumor-bearing mice

After obtaining approval by the Animal Care and Use Committee of Okayama University, all animal experiments were performed according to the Principles of Laboratory Animal Care (NIH publication #85-23).

Mouse breast cancer 4T1 cells (CRL-2539), purchased from American Type Culture Collection (Rockville, MD, USA), were cultured in RPMI-1640 medium containing FBS (10%), gentamycin (20 µg/mL), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere of 5% CO₂/95% air.

A tumor-bearing model mouse was established by subcutaneous injection of 4T1 cells (2.5×10^6) into the left flank of 5–6-week-old male BALB/c mice (Charles River Laboratories Japan Inc., Yokohama, Kanagawa, Japan). The tumor diameter was measured using calipers, and tumor volume was calculated using the following equation [19]:

$$\text{Tumor volume (mm}^3\text{)} = (\text{Long diameter}) \times (\text{Short diameter})^2 \times 0.52$$

The experimental schedule is schematically summarized in Fig. 1. To evaluate the anti-tumor effect, when the tumor grew to approximately 100 mm³, PE-SU5416 (10 mg/kg as SU5416) was intravenously injected on “day 0”. For sequential administration, PL-PTX (1 mg/kg as PTX) was injected intravenously 24 h after PE-SU5416 administration (day

1). Sequential administration was repeated up to three times at 48-h intervals. In details, for one sequential administration (Seq×1), PE-SU5416 and PL-PTX was administered on days 0 and 1, respectively. For two (Seq×2) and three (Seq×3) sequential administrations, PE-SU5416 and PL-PTX was consecutively administered on days 3 and 4, and days 6 and 7, respectively. To evaluate the three administrations of PE-SU5416 (PE-SU5416×3) or PL-PTX (PL-PTX×3) alone, each formulation was administered according to the dosing schedule described in Fig. 1.

For experiments regarding with immunofluorescent analysis, detection of vessels with blood flow, liposomal distribution, tumor microenvironment, PE-SU5416 was administered when tumor grew to approximately 400–500 mm³ (Fig. 1).

2.4. Immunofluorescence analysis

The mice were euthanized under anesthesia on days 2, 5, and 8 (Fig. 1). For detecting CD31 (a vascular endothelial cell marker), NG2 (a pericyte marker), and carbonic anhydrase IX (CA9, a hypoxia marker), tumor sections (10 μm-thick) were prepared as described previously [14], and fixed with ice-cold acetone. For detecting α-SMA (a CAF marker) and collagen I, mice were transcardially perfused with 4% paraformaldehyde (PFA)-PBS and tumor tissues excised from mice were immersed in 4% PFA-PBS for 4 h

at 4°C. Next, tumor sections (10 µm-thick) prepared as described previously [14], were further fixed with 4% PFA-PBS for 20 min at RT.

After blocking with 5% FBS-PBS for 2 h, tumor sections were incubated with primary antibodies overnight at 4°C followed by incubation with appropriate secondary antibodies for 1 h at RT. The antibodies used in this study are listed in Supplementary Table 1.

Fluorescence images were captured using a fluorescence microscope (Biozero BZ-X700, Keyence, Osaka) at the Central Research Laboratory of the Okayama University Medical School. Whole-tumor section images were reconstructed using the image joint software BZ Analyzer (Keyence). The peripheral region was defined as approximately <700 µm from the edge of the tumor section and the core region was defined as the remaining area (approximately >700 µm from the edge). The fluorescence-positive area, merged area, and tumor area were measured using Image J software.

2.5. Evaluation of vessels with blood flow and liposomal distribution in tumor tissues

DiI-labeled PEG-liposomes (40 µmol total lipids/kg) were intravenously administered to the mice on days 2, 5, or 8 at one, two, or three sequential administrations, respectively (Fig. 1). To assess vessels with blood flow or intratumoral distribution, tumor tissues were excised from mice 1 or 24 h after the administration of DiI-labeled PEG-liposomes,

respectively. To detect CD31, tumor sections (10 μ m-thick), prepared as described previously [14], were fixed with acetone, blocked with 5% FBS-PBS for 2 h, and incubated with primary antibodies overnight at 4°C followed by incubation with appropriate secondary antibodies for 1 h at RT. The antibodies used in this study are listed in Supplementary Table 1.

2.6. Enzyme-linked immunosorbent assay (ELISA) of transforming growth factor- β (TGF- β)

Tumors of approximately 400–500 mm³ were excised from mice and cut into small pieces with scissors and homogenized using a Power Masher II homogenizer (Nippi Inc., Tokyo) for 5 min in RIPA buffer containing Tris-HCl (50 mM, pH7.5), EDTA (1 mM), sodium chloride (150 mM), sodium dodecyl sulfate (0.1%), sodium deoxycholate (0.5%), and Nonidet P40-substitute (1%) (Nacalai Tesque, Kyoto, Japan) with protease inhibitor cocktail (cOmplete Mini, Sigma-Aldrich). The homogenates were then gently agitated on a rocker shaker at 4°C for 2 h and centrifuged at 11,000 $\times$ g for 20 min. The amount of TGF- β in each sample was determined using Mouse TGF- β sandwich ELISA kit (Proteintech, Rosemont, IL, USA), following the manufacturer's instructions. The results were normalized to the protein levels in tissue lysates, which were determined using a

DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Statistical analysis

Results are expressed as the mean with standard deviation (S.D.) of more than three experiments. Statistical analysis was performed by a one-way analysis of variance (ANOVA), followed by Tukey's post-hoc multiple comparison test. For anti-tumor activity, data were analyzed by two-way repeated-measures ANOVA post-hoc Bonferroni test.

3. Results and Discussion

3.1. Anti-tumor effect of repeated sequential administration of PE-SU5416 and PL-PTX in 4T1 tumor-bearing mice

To seek higher anti-tumor effect by vascular normalization therapy, we examined the effect of the repeated sequential administration of PE-SU5416 and PL-PTX in 4T1 tumor-bearing mice. First, preliminarily, the anti-tumor effect of Seq×2 was examined, but the tumor growth profile did not significantly change compared to that of Seq×1 (data not shown). We then investigated the effect of Seq×3 following the dosing schedule shown in Fig. 1. As shown in Fig. 2, Seq×3 suppressed tumor growth more significantly than the

control or Seq×1 groups from day 8 or 16, respectively. Seq×1 significantly suppressed tumor growth from day 12 to approximately 59% of the control on day 21, which was consistent with our previous results [14]. In contrast, tumor growth in the Seq×3 group was suppressed to approximately 26% of that in the control group and 44% of that in the Seq×1 group on day 21 (Fig. 2). Furthermore, PE-SU5416×3 or PL-PTX×3 did not significantly suppress tumor growth, and the Seq×3 group did also reveal a significant difference in tumor growth compared to the PE-SU5416×3 or PL-PTX×3 groups from day 9 or day 11, respectively. These results indicate that three sequential administrations of PE-SU5416 and PL-PTX is a better regimen and should be the first step for the remission based on the vascular normalization therapy.

3.2. Structural evaluation of tumor vasculatures

To elucidate the reason for the higher anti-tumor activity by Seq×3, we evaluated the effects of Seq×1, Seq×2, and Seq×3 on the structural properties of tumor vessels and compared the results with those of PE-SU5416×1, PE-SU5416×2, and PE-SU5416×3. Isolated tumor sections were immunostained for the pericyte marker NG2 and the endothelial cell marker CD31. The pericyte-covered vascular endothelial cells were shown as merge of NG2 and CD31 in the core (Fig. 3(A)) and peripheral (Fig. 3(B))

regions. Quantitative results indicated that CD31⁺ area was not changed in the PE-SU5416 or sequential administration groups compared to the control group, except for core regions in the PE-SU5416×2 and Seq×2 groups, suggesting that PE-SU5416 did not reduce tumor vascular endothelial cells under all dosing conditions (Fig. 4(A)). In the case of pericytes, increasing tendencies in NG2⁺ area were observed in both the peripheral and core regions under all dosing conditions; specifically, PE-SU5416×1 and Seq×1 significantly increased NG2⁺ area in the peripheral region (Fig. 4(B)). Merged area of CD31⁺ and NG2⁺, reflecting the area of pericyte-covered vascular endothelial cells, revealed the increasing tendency in the area in PE-SU5416×1, PE-SU5416×2, Seq×1, and Seq×2 groups (Fig. 4(C)). Specifically, PE-SU5416×1 and PE-SU5416×2 significantly increased the area of pericyte-covered vascular endothelial cells in the core region. In contrast, a slight decrease in area was observed by PE-SU5416×3 and Seq×3. The ratio of pericyte-covered vascular endothelial cells to total vascular endothelial cells was significantly increased by PE-SU5416×1, PE-SU5416×2, Seq×1, and Seq×2 (Fig. 4(D) and Supplementary Table 2). Since an increase in the ratio indicates an increase in pericyte-covered vascular endothelial cells, it is clear that PE-SU5416 or sequential administration of PE-SU5416 and PL-PTX can structurally normalize tumor vasculatures for up to two administrations. However, structural improvement was not sustained in PE-

SU5416×3 or Seq×3 groups.

In this study, there were no differences in improvement in vascular structure outcomes between the PE-SU5416 alone and sequential administration groups (Fig. 4), suggesting that PL-PTX itself did not influence the structure of tumor vasculatures or impair the vasculature structurally improved by PE-SU5416. Regarding the effect of PE-SU5416 on tumor vascular structure, we also observed an increasing tendency of VE-cadherin, an endothelial-specific adhesion molecule [20], in tumor vasculatures until after two administrations of PE-SU5416 with a significant difference in the core region of PE-SU5416×1 group (Supplementary Fig. 1). This result also supports the efficacy in structural normalization of tumor vasculatures by PE-SU5416.

Normalization of tumor vasculature by anti-angiogenic agent is known to be a dose-dependent and transient effect, and requires an optimal balance between pro- and anti-angiogenic factors [7,21]. In terms of vascular structure, our results suggest that PE-SU5416×1 and PE-SU5416×2, and Seq×1 and Seq×2 treatments maintain an adequate pro- and anti-angiogenic balance; however, PE-SU5416×3 and Seq×3 treatments were likely to shift the balance toward anti-angiogenesis.

Because the highest anti-tumor activity was observed in the Seq×3 group (Fig. 2), tumor vasculatures should be examined based on their functional aspects. Therefore, the

effect of repeated sequential administration on the functional properties of tumor vasculatures was investigated.

3.3. Functional evaluation of tumor vasculatures

First, hypoxic regions in tumor tissues were estimated by immunostaining for CA9, a marker of hypoxia [22] (Fig. 5); CD31 was also immunostained to identify the location of endothelial cells. While fluorescence derived from CA9 was observed throughout the tumor tissues in the control group, PE-SU5416 alone or sequential administration clearly decreased fluorescence under all dosing conditions (Fig. 5(A)). Quantitative analysis of the images indicated that PE-SU5416 alone or sequential administration significantly decreased the hypoxic region under all dosing conditions, whereas the area tended to increase in the control group (Fig. 5(B)(i)). The CA9⁺ area normalized to the tumor area clearly indicates that two and three administrations significantly reduced the hypoxic region further in both PE-SU5416 alone and sequential administration groups (Fig. 5(B)(iii) and Supplementary Table 3), suggesting that oxygen supply to tumor tissues through the tumor vasculature was significantly improved and that this improvement was sustained until after PE-SU5416×3 and Seq×3.

Next, to determine tumor vasculature with blood flow (functional tumor vasculature),

the intratumoral distribution of DiI-labeled PEG-liposomes was examined 1 h after the intravenous administration (Fig. 6). The representative images of the DiI⁺ area clearly indicate that DiI-labeled PEG-liposomes were rarely distributed to tumor tissues, whereas PE-SU5416 alone and sequential administration caused a substantial intratumoral distribution of DiI-labeled PEG-liposomes (Fig. 6(A)). Calculated using CD31⁺ area (Fig. 6(B)(i)), DiI⁺ area (Fig. 6(B)(ii)), and the merged area of DiI⁺ and CD31⁺ indicating tumor vessels with blood flow (Fig. 6(B)(iii)), the ratio of tumor vessels with blood flow to whole tumor vessels (Merge area/CD31⁺ area) (Fig. 6(B)(iv)) was significantly increased in the PE-SU5416 alone or sequential administration groups under all dosing conditions. This functional improvement was maintained until after three administrations, leading to the highest improvement in PE-SU5416×3 (approximately 7-fold) and Seq×3 (approximately 6-fold) (Supplementary Table 4) groups, compared to the control group.

From the aforementioned results, tumor vasculature was confirmed to be functionally improved until after PE-SU5416×3 and Seq×3. We then investigated whether functional improvement in the tumor vasculature leads to an improvement in the intratumoral distribution of PEG-liposomes. Fig. 7(A) shows typical images of the intratumoral distribution of DiI-labeled-PEG-liposomes 24 h after intravenous administration, where CD31 was also immunostained to identify the location of endothelial cells. DiI⁺ area

normalized to tumor area (Fig. 7(B)(iii)), calculated based on the quantitative analysis of images for DiI⁺ area (Fig. 7(B)(i)) and tumor area (Fig. 7(B)(ii)), clearly indicated that the intratumoral distribution of DiI-labeled-PEG-liposomes increased under all dosing conditions and that the improving effect compared to the control group was the highest in three-time administration groups (approximately 5.3-fold) (Fig. 7(B)(iii), Supplementary Table 5).

Taken together, the tumor vasculature was functionally improved until after PE-SU5416×3 and Seq×3, and the improvement led to enhanced intratumoral distribution of PEG-liposomes under all dosing conditions. Furthermore, PL-PTX did not significantly affect the functional improvement induced by PE-SU5416.

The functional evaluation of tumor vasculature above (Figs. 5, 6, and 7) showed some discrepancy to the structural evaluation (Figs. 3 and 4); in other words, the tumor vasculatures were structurally normalized until after PE-SU5416×2 and Seq×2, but functionally improved until after PE-SU5416×3 and Seq×3. The result that Seq×3 exhibited the highest anti-tumor effect in mice bearing 4T1 tumor (Fig. 2) also suggests the maintenance of functional improvement until after Seq×3. We then attempted to elucidate the reasons for this by focusing on the tumor microenvironment.

3.4. Evaluation of tumor microenvironment

CAFs are major components of the tumor microenvironment and play important roles in tumor growth and progression [23–26]. They also produce abundant ECM, including collagen [27], which elevates interstitial fluid pressure (IFP) and prevents the distribution and diffusion of drugs into tumor tissues [28,29]. We previously indicated that a single administration of PE-SU5416 reduced CAFs and collagen in tumor tissues, which could be associated with an improvement in the intratumoral distribution of subsequently administered PL-PTX and anti-tumor activity [14]. Therefore, we evaluated the effects of repeated sequential administration on the tumor microenvironment. Fig. 8(A), (B), and (C) shows typical images of whole tumor sections, core, and peripheral regions, respectively, immunostained for the CAF marker α -SMA, and the major ECM component collagen, after PE-SU5416 $\times$ 1, PE-SU5416 $\times$ 2, and PE-SU5416 $\times$ 3 or Seq $\times$ 1, Seq $\times$ 2, and Seq $\times$ 3. Figure 9 shows the results of the quantitative analysis of the images. PE-SU5416 $\times$ 1 or Seq $\times$ 1 did not change the α -SMA⁺ area, but PE-SU5416 $\times$ 2 and PE-SU5416 $\times$ 3 or Seq $\times$ 2 and Seq $\times$ 3 significantly decreased it (Fig. 9(A)(i)). Furthermore, the detailed analysis for the core (Fig. 9(A)(ii)) and peripheral (Fig. 9(A)(iii)) regions of tumor sections also indicated that the α -SMA⁺ area was significantly decreased in both the core and peripheral regions by PE-SU5416 $\times$ 2 and PE-SU5416 $\times$ 3 or Seq $\times$ 2 and Seq $\times$ 3,

but not by PE-SU5416×1 or Seq×1 (Fig. 9(A)(ii) and (iii) and Supplementary Table 6). Similar to CAFs, the collagen⁺ area was significantly decreased by PE-SU5416×2 and PE-SU5416×3 or Seq×2 and Seq×3, but not by PE-SU5416×1 or Seq×1 (Fig. 9(B)(i)). A detailed analysis also indicated a significant reduction in the collagen⁺ area in the peripheral region by PE-SU5416×2 and PE-SU5416×3 or Seq×2 and Seq×3 (Fig. 9(B)(iii) and Supplementary Table 7), but a reduction in the core region was observed only by PE-SU5416×2 and Seq×2 (Fig. 9(B)(ii) and Supplementary Table 7). PE-SU5416×1 or Seq×1 did not affect the amount of collagen in any region (Fig. 9(B)(ii) and (iii)). Because the changes in collagen were generally associated with those in CAFs (Fig. 9), we postulated that repeated PE-SU5416 or sequential administrations caused a reduction in CAFs, which subsequently led to the suppression of collagen production in the tumor tissues. Unexpectedly, the effects of the single administration on CAFs and collagen were different from our previous results (Maruyama et al., 2024); however, the single administration might have been insufficient to exert this effect, and the results obtained might have been poorly reproducible. Nonetheless, the results obtained after repeated administration confirmed that PE-SU5416 and sequential administrations significantly and reproducibly reduced the levels of CAFs and collagen in tumor tissues. Furthermore, although there was a significant difference in the α -SMA⁺ area between the

two-time administration groups in peripheral region (Fig. 9(A)(iii) and (B)(iii)), the effects of PE-SU5416 on CAFs and collagen were almost maintained in the sequential administration group (Fig. 9(A)(i) and (B)(i)), suggesting that changes in CAFs and subsequent changes in collagen would be caused by PE-SU5416, with PL-PTX not having a considerable effect.

As described above, because CAFs and collagen enhance IFP and in turn suppress the intratumoral distribution of drugs, including particulate formulation such as liposomes [28,29], the reduction in CAFs and collagen caused by repeated PE-SU5416 or sequential administrations would have caused IFP reduction, which would have compensated for the balance in vascular structure shifting toward the anti-angiogenesis and would have led to the facilitation of intratumoral distribution of DiI-labeled PEG-liposomes (Figs. 6 and 7). Therefore, the intratumoral distribution of PL-PTX administered after the second and third administration of PE-SU5416 would also have been facilitated, resulting in the stronger anti-tumor effects observed in Fig. 2.

However, the mechanism by which PE-SU5416 treatment reduces CAFs is still unknown; therefore, we attempted to elucidate it.

3.5. Involvement of TGF- β in tumor microenvironment

Various cell types, such as pericytes, adipocytes, endothelial cells, and bone marrow-derived mesenchymal stem cells, are considered the origin of CAFs [30,31]; however, it is believed that most CAFs are derived from tissue-resident fibroblasts [32,33]. TGF- β is widely accepted as one of the main activators of resting fibroblasts becoming CAFs [34–37]. Therefore, we determined TGF- β in tumor tissues by ELISA after repeated sequential administration. Two and three administrations of PE-SU5416 alone or sequential administrations significantly decreased TGF- β in tumor tissues to approximately 56% to 72% of the control (Fig. 10 and Supplementary Table 8). These results were comparable with those for CAFs shown in Fig. 9(A) and Supplementary Table 6, suggesting that the reduction in TGF- β is responsible for the decrease in CAFs in tumor tissues. Sequential administration of PL-PTX did not significantly change the effect of PE-SU5416 on TGF- β , indicating that the reduction in TGF- β levels in tumor tissues was caused by PE-SU5416.

The reason why TGF- β was reduced by PE-SU5416 is not clear yet. However, since it was reported that a hypoxic environment induces TGF- β expression [38,39], improvement in oxygen supply to tumor tissue following PE-SU5416 or sequential administrations (Fig. 5) might have led to the suppression of TGF- β expression.

In the current study, three administrations of PE-SU5416 with PL-PTX resulted in a

significant improvement in the anti-tumor effect. Because PE-SU5416 is a PEG-modified nanoemulsion that can achieve efficient tumor delivery based on enhanced permeability and retention effect, the dose and frequency of administration were low compared to other studies using SU5416 [10–12,40], suggesting that the risk of side effects from repeated PE-SU5416 administration would be low.

In our series of studies, male mice have been used for establishing 4T1 tumor-bearing mice to eliminate the influence of sex differences and to rigorously compare the therapeutic effects with those in colon-26 tumor-bearing mice. In addition, another group reported the similarities in timing of tumor development, necrosis, lung metastases, and histological features of tumor tissues between male and female BALB/c mice bearing orthotopically transplanted 4T1 tumors [41], suggesting that repeated sequential administration would also be effective in female mice. However, it is necessary to examine therapeutic efficacy of vascular normalization therapy in female mice with orthotopically implanted 4T1 cells for future clinical application in breast cancer treatment.

4. Conclusion

Three sequential administrations of PE-SU5416 and PL-PTX exhibited higher anti-

tumor activity compared to one or two sequential administrations. PE-SU5416 structurally normalized tumor vasculatures until after the second administration, but improved tumor vasculature functions such as oxygen supply, blood flow, and liposomal distribution until after the third administration. Furthermore, two and three PE-SU5416 administrations reduced CAFs, caused by decreased TGF- β , leading to a subsequent decrease in collagen in 4T1 tumor tissues. The decrease in CAFs and collagen would have contributed to the functional improvement in tumor vasculatures by suppressing IFP, leading to enhanced intratumoral distribution and anti-tumor activity of PL-PTX in 4T1 tumor-bearing mice. The findings of the current study provide useful insights for the clinical application of vascular normalization strategies utilizing particulate formulations such as PE-SU5416 and PL-PTX. Our findings also suggest that the vascular normalization strategy using SU5416 could facilitate the tumor distribution of macromolecular drugs, and therefore, the strategy might be applied for nucleic acid-loaded lipid nanoparticles, macromolecules such as immune checkpoint inhibitors, and/or antibody-drug conjugates.

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CRedit authorship contribution statement

Masato Maruyama: Conceptualization, Methodology, Investigation, Writing-Original Draft, Funding acquisition, Formal Analysis, Writing-review & editing. **Reiya Torii:** Formal Analysis, Investigation. **Hazuki Matsui:** Formal Analysis, Investigation. **Hiroki Hayashi:** Formal Analysis, Investigation. **Ken-ichi Ogawara:** Conceptualization, Methodology. **Kazutaka Higaki:** Conceptualization, Methodology, Investigation, Writing-Original Draft, Review & Editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online

Figure legends

Fig. 1. Experimental schedule of repeated sequential administration of PE-SU5416 and PL-PTX.

Day 0 was defined as the day when the tumor volume reached approximately 100 mm³ for evaluation of anti-tumor effect or 400–500 mm³ for evaluation of structure and function of tumor vessels, and tumor microenvironment. Seq×1, Seq×2, and Seq×3 mean one, two, and three sequential administrations of PE-SU5416 and PL-PTX. PE-SU5416 or PL-PTX was intravenously injected at each indicated time point. PE-SU5416×3 or PL-PTX×3 represents three administrations of PE-SU5416 or PL-PTX, respectively.

Fig. 2. Evaluation of anti-tumor effect of repeated sequential administration of PE-SU5416 and PL-PTX in 4T1 tumor-bearing mice.

Doses of PE-SU5416 and PL-PTX were 10 mg/kg as SU5416 and 1 mg/kg as PTX, respectively. PE-SU5416×3 or PL-PTX×3 means three administrations of PE-SU5416 or PL-PTX, respectively. Seq×1 or Seq×3 represents one or three sequential administrations of PE-SU5416 and PL-PTX. Keys: ○, ctrl (untreated control); △, PE-SU5416×3; □, PL-PTX×3; ◇, Seq×1; ◆, Seq×3. Results are expressed as the mean with S.D. bars (n=4–5).

***p<0.001, *p<0.05 compared with ctrl group. †††p<0.001, ††p<0.01, †p<0.05 compared

with PE-SU5416×3 group. ^{***}p<0.001, ^{**}p<0.01 compared with PL-PTX×3 group. ^{\$\$\$}p<0.001, ^{\$}p<0.05 compared with Seq×1 group.

Fig. 3. Immunofluorescence images for pericytes and vascular endothelial cells in 4T1 tumor tissues after repeated sequential administration of PE-SU5416 and PL-PTX.

Tumor sections, prepared after one (×1), two (×2), and three (×3) administrations of PE-SU5416 (SU) or PE-SU5416-PL-PTX (Seq), were immunostained for pericyte (NG2, green) and endothelial cells (CD31, red). Ctrl means an untreated control. Representative merged images for each group in the core (A) and peripheral (B) regions were shown. Scale bars, 100 μm.

Fig. 4. Quantitative analysis of pericyte coverage for vascular endothelial cells in 4T1 tumor tissue after repeated sequential administration of PE-SU5416 and PL-PTX.

(A) CD31⁺ area, (B) NG2⁺ area, (C) merged area of CD31⁺ and NG2⁺, and (D) the ratio of NG2⁺CD31⁺ merged area/CD31⁺ area in all, the core, and peripheral regions of each group. “All” indicates the average of the core and peripheral regions. Ctrl, SU and Seq

mean untreated control, PE-SU5416-treated (SU; $\times 1$, $\times 2$, $\times 3$), and sequentially treated (Seq; $\times 1$, $\times 2$, $\times 3$) groups of mice, respectively. Images of total 23–25 fields (taken with objective $\times 20$) from 5 mice were analyzed for each group in the core and peripheral regions. Results are expressed as the mean with S.D. bars (n=5). **p< 0.01, *p< 0.05 compared with each ctrl group.

Fig. 5. Effect of repeated sequential administration of PE-SU5416 and PL-PTX on hypoxic regions in 4T1 tumor tissues.

(A) Representative images of whole tumor sections after one ($\times 1$), two ($\times 2$), and three ($\times 3$) intravenous administrations of PE-SU5416 (SU), PE-SU5416-PL-PTX (Seq), or untreated control (ctrl). Immunostaining were performed for hypoxic region (CA9, green) and endothelial cells (CD31, red). Scale bars, 2 mm. (B) (i) CA9⁺ area, (ii) tumor area, and (iii) the ratio of CA9⁺ area/tumor area in each group were shown. Results are expressed as the mean with S.D. bars (n=3–5). ***p< 0.001, **p< 0.01, *p< 0.05 compared with each ctrl group.

Fig. 6. Effect of repeated sequential administration of PE-SU5416 and PL-PTX on vessels with blood flow in 4T1 tumor tissues.

(A) Representative images of whole tumor sections at 1 h after intravenous administration of DiI-labeled PEG-liposomes to untreated control (ctrl), PE-SU5416-treated (SU; $\times 1$, $\times 2$, $\times 3$), and sequentially treated (Seq; $\times 1$, $\times 2$, $\times 3$) groups of mice. Merged area (yellow) of DiI-labeled PEG-liposomes (red) and endothelial cells (CD31, green) shows vessels with blood flow. Scale bars, 1 mm. (B) (i) CD31⁺ area, (ii) DiI⁺ area, (iii) merged area, and (iv) the ratio of CD31⁺ DiI⁺ merged area/CD31⁺ area. Results are expressed as the mean with S.D. bars (n=4–5). **p< 0.01, *p< 0.05 compared with each ctrl group.

Fig. 7. Effect of repeated sequential administration of PE-SU5416 and PL-PTX on disposition of PEG-liposomes in 4T1 tumor tissues.

(A) Representative images of whole tumor sections at 24 h after intravenous administration of DiI-labeled PEG-liposomes to untreated control (ctrl), PE-SU5416-treated (SU; $\times 1$, $\times 2$, $\times 3$), and sequentially treated (Seq; $\times 1$, $\times 2$, $\times 3$) groups of mice. DiI-labeled PEG-liposomes, red; endothelial cells (CD31), green. Scale bars, 2 mm. (B) (i) DiI⁺ area, (ii) tumor area, and (iii) the ratio of DiI⁺ area/tumor area. Results are expressed as the mean with S.D. bars (n=3–5). **p< 0.01, *p< 0.05 compared with each ctrl group.

Fig. 8. Immunofluorescence images for CAF and collagen I in 4T1 tumor tissues after repeated sequential administration of PE-SU5416 and PL-PTX.

Tumor sections, prepared after one ($\times 1$), two ($\times 2$), and three ($\times 3$) administrations of PE-SU5416 (SU) or PE-SU5416-PL-PTX (Seq), were immunostained for cancer-associated fibroblasts (α -SMA, green) and collagen I (red). Ctrl means an untreated control. (A) Representative images of whole tumor sections. Scale bars, 2 mm. Representative magnified images for each group in the core (B) and peripheral (C) regions in 4T1 tumor tissue. Scale bars, 100 μ m.

Fig. 9. Quantitative analysis for CAF and collagen I in 4T1 tumor tissue after repeated sequential administration of PE-SU5416 and PL-PTX.

(A) α -SMA⁺ area in whole tumor sections (i), in the core (ii) and peripheral (iii) regions in each group. (B) Collagen I⁺ area in whole tumor sections (i), in the core (ii) and peripheral (iii) regions in each group. Ctrl, SU and Seq mean untreated control, PE-SU5416-treated (SU; $\times 1$, $\times 2$, $\times 3$), and sequentially treated (Seq; $\times 1$, $\times 2$, $\times 3$) groups of mice, respectively. Images of total 20–25 fields (taken with objective $\times 20$) from 4–5 mice were analyzed for each group in the core and peripheral regions. Results are expressed as the mean with S.D. bars (n=4–5). ***p< 0.001, **p< 0.01, *p< 0.05 compared with each

ctrl group. [†]p< 0.05 compared with SU×2 group.

Fig. 10. Effect of repeated sequential administration of PE-SU5416 and PL-PTX on TGF-β in 4T1 tumor tissues.

The amount of TGF-β in 4T1 tumor tissues after one (×1), two (×2), and three (×3) administrations of PE-SU5416 (SU), PE-SU5416-PL-PTX (Seq) was determined by ELISA. Ctrl means an untreated control. Results are expressed as the mean with S.D. bars (n=4–5). ***p< 0.001; **p< 0.01; *p< 0.05 compared with each ctrl group.

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Figure 1 Maruyama et al.

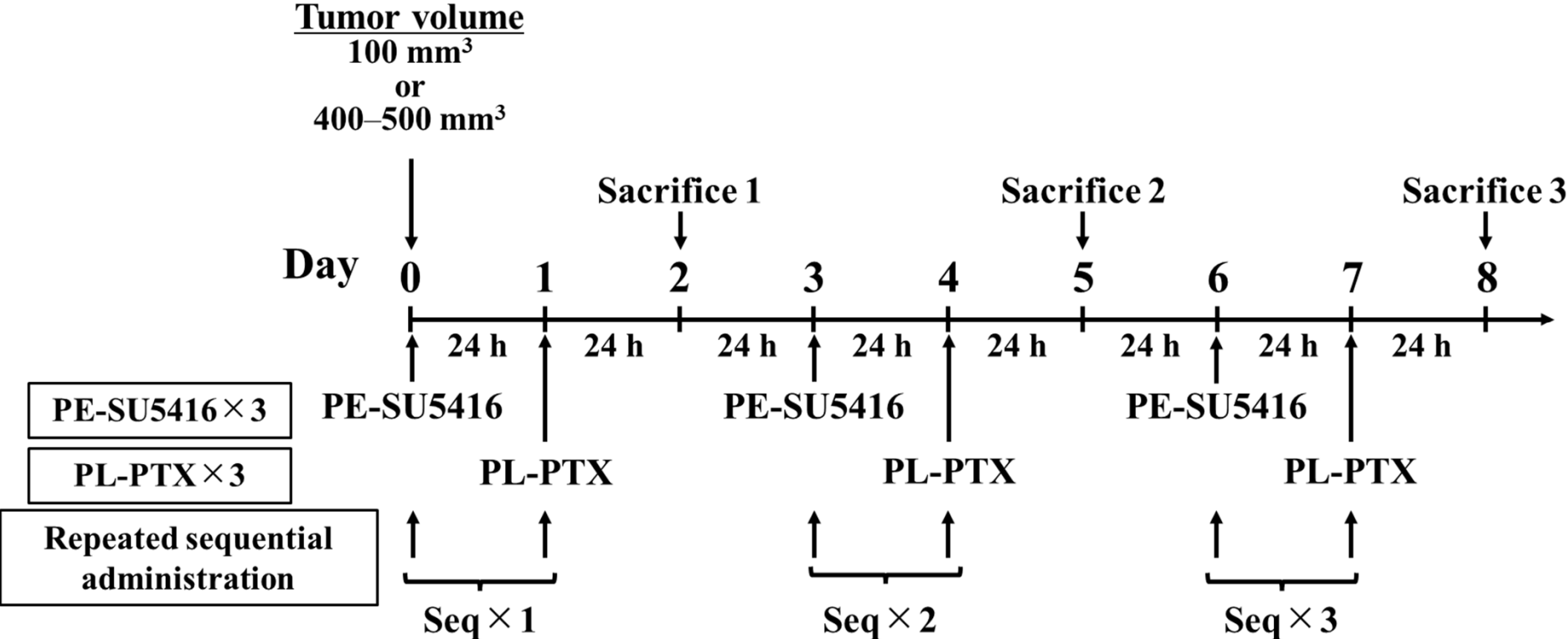


Figure 2 Maruyama et al.

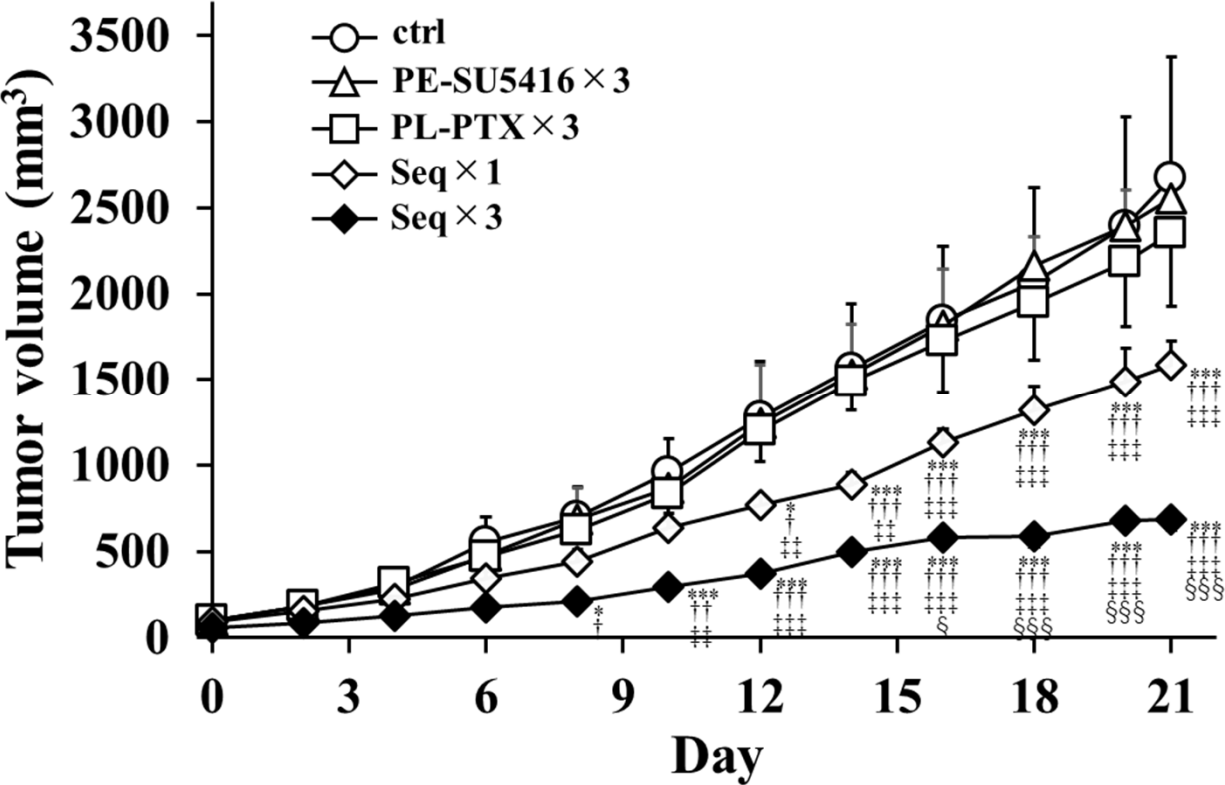
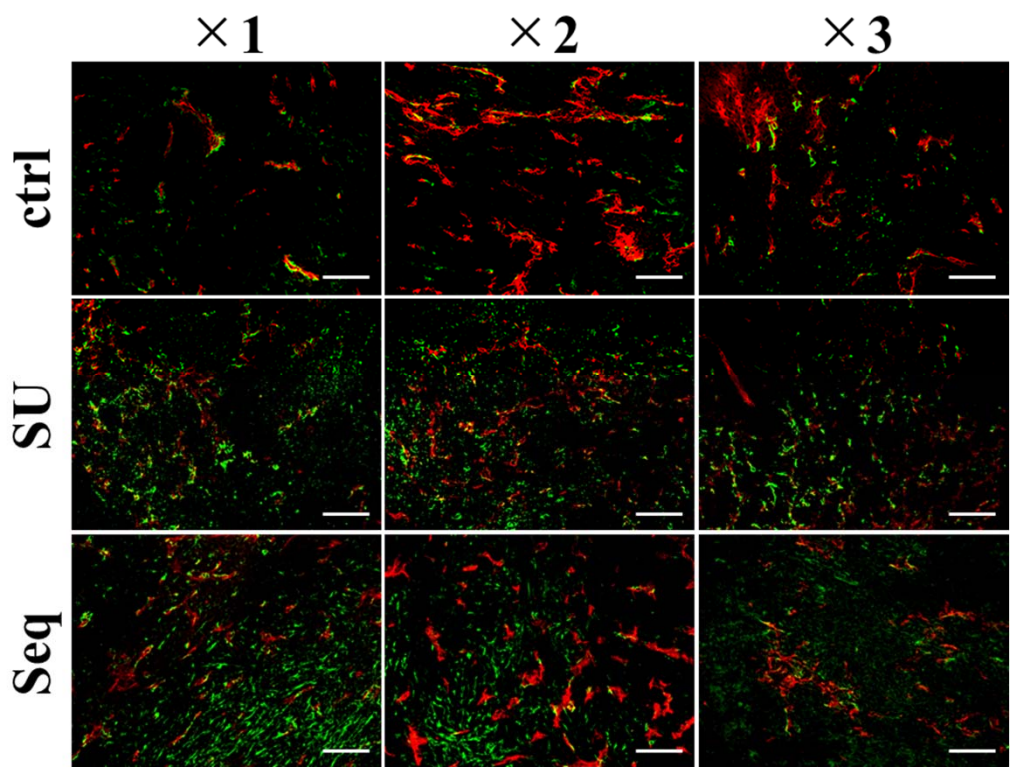


Figure 3 Maruyama et al.

(A) Core



(B) Peripheral

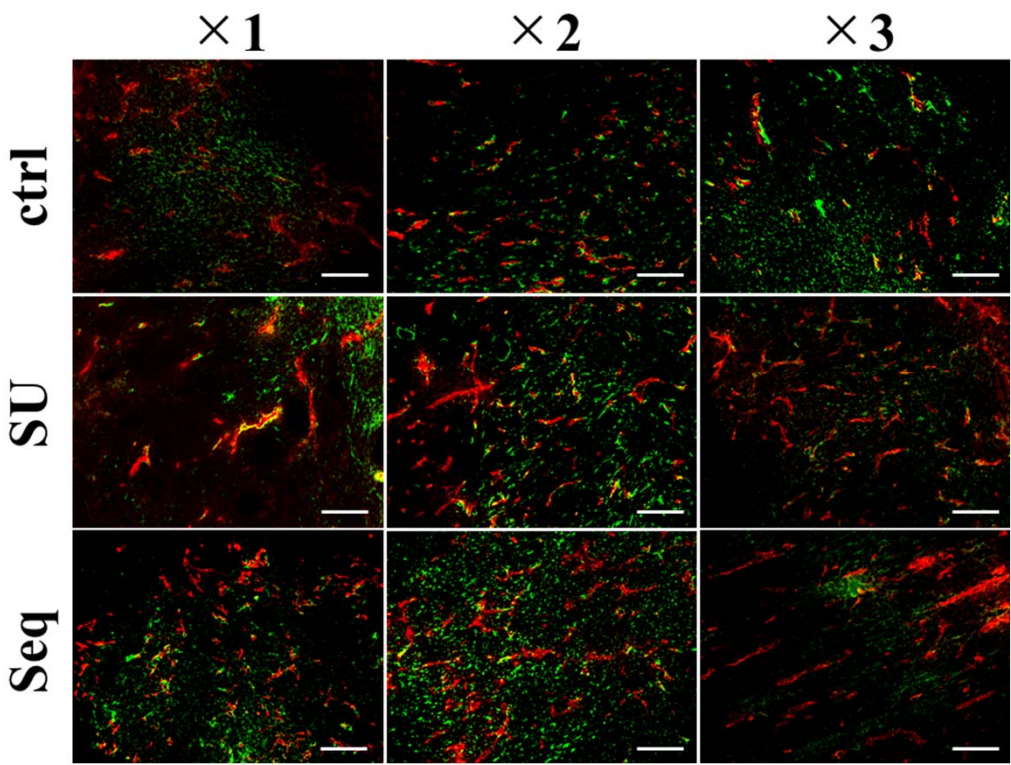


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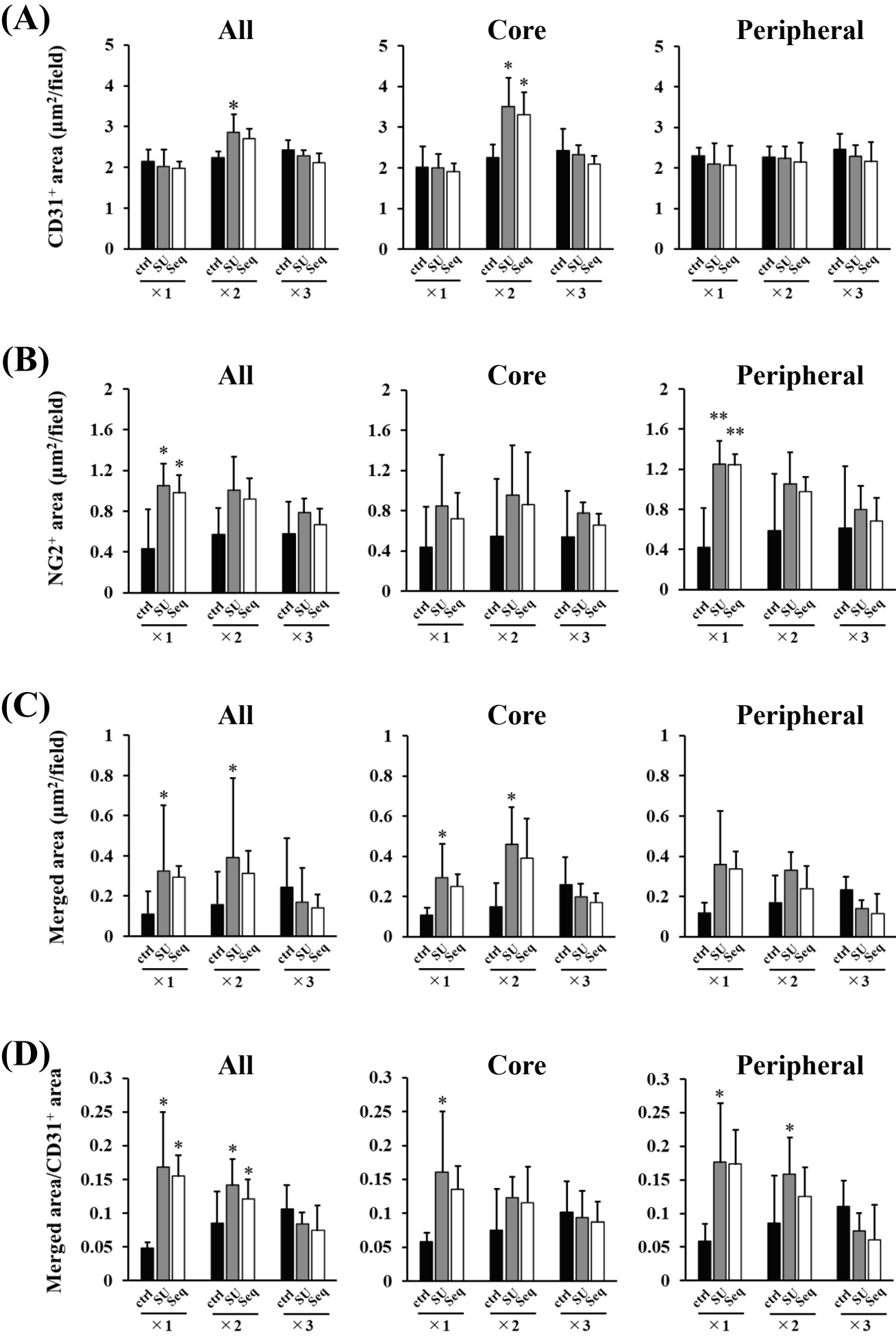


Figure 5 Maruyama et al.

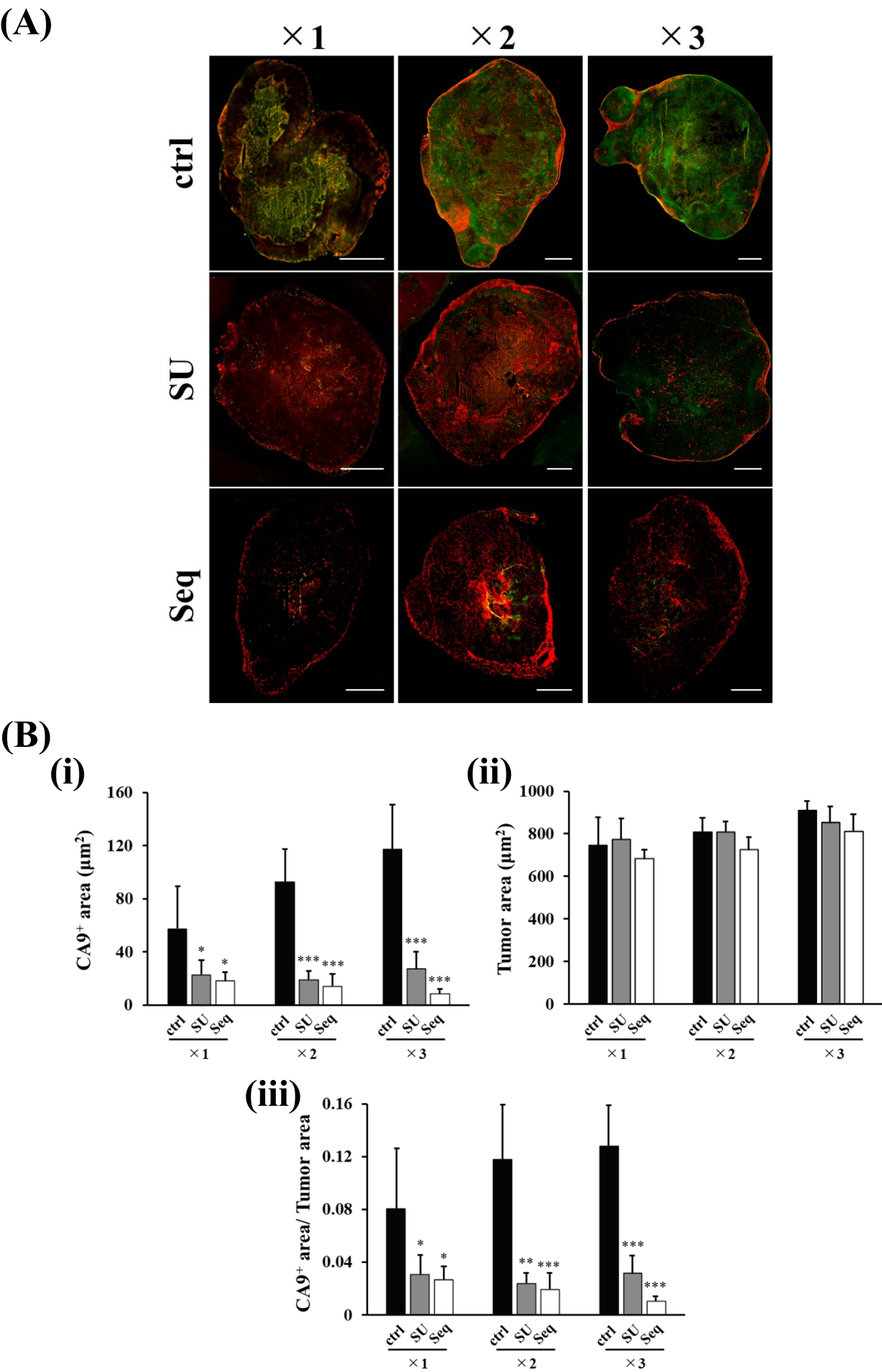


Figure 6 Maruyama et al.

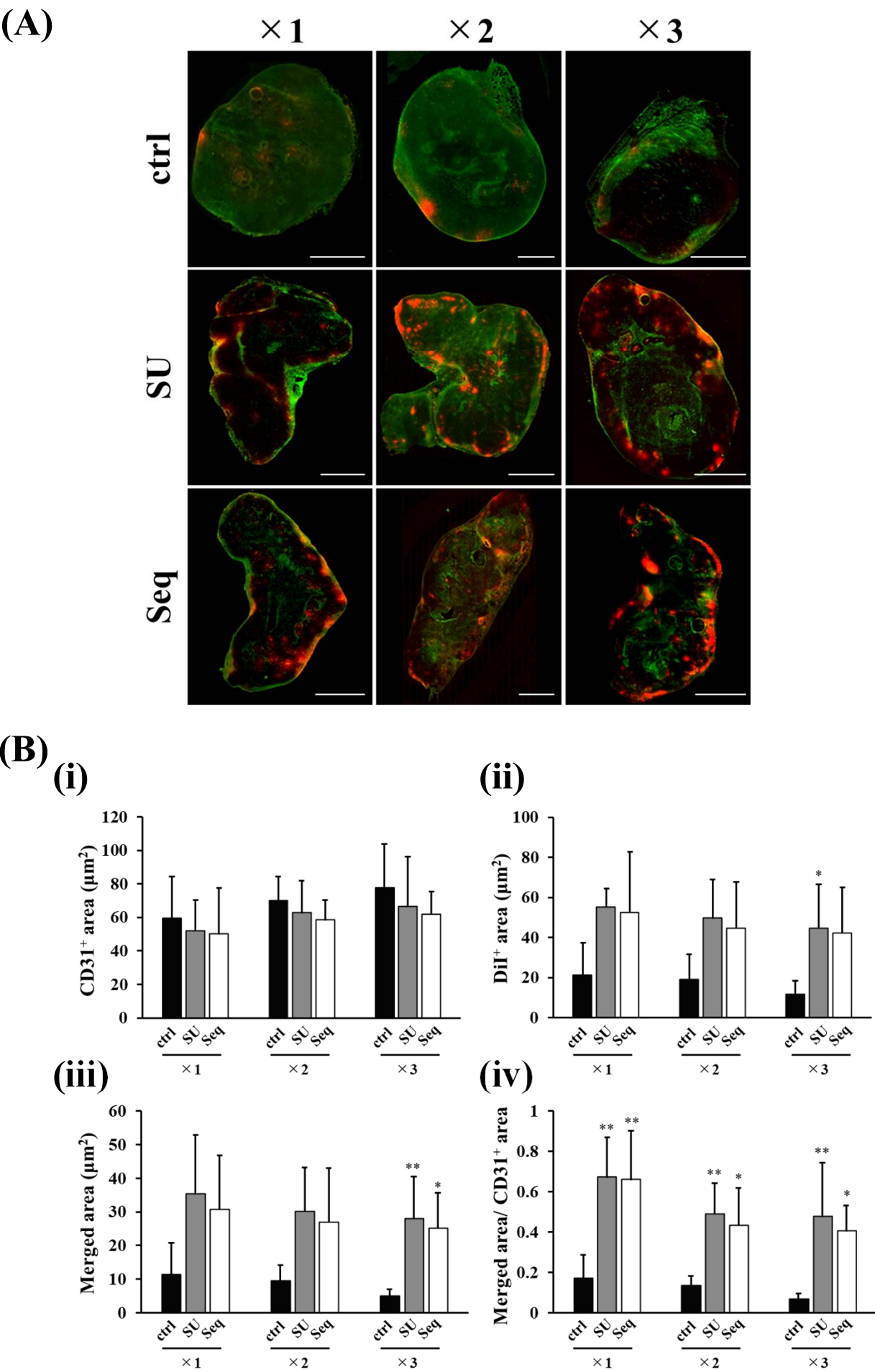
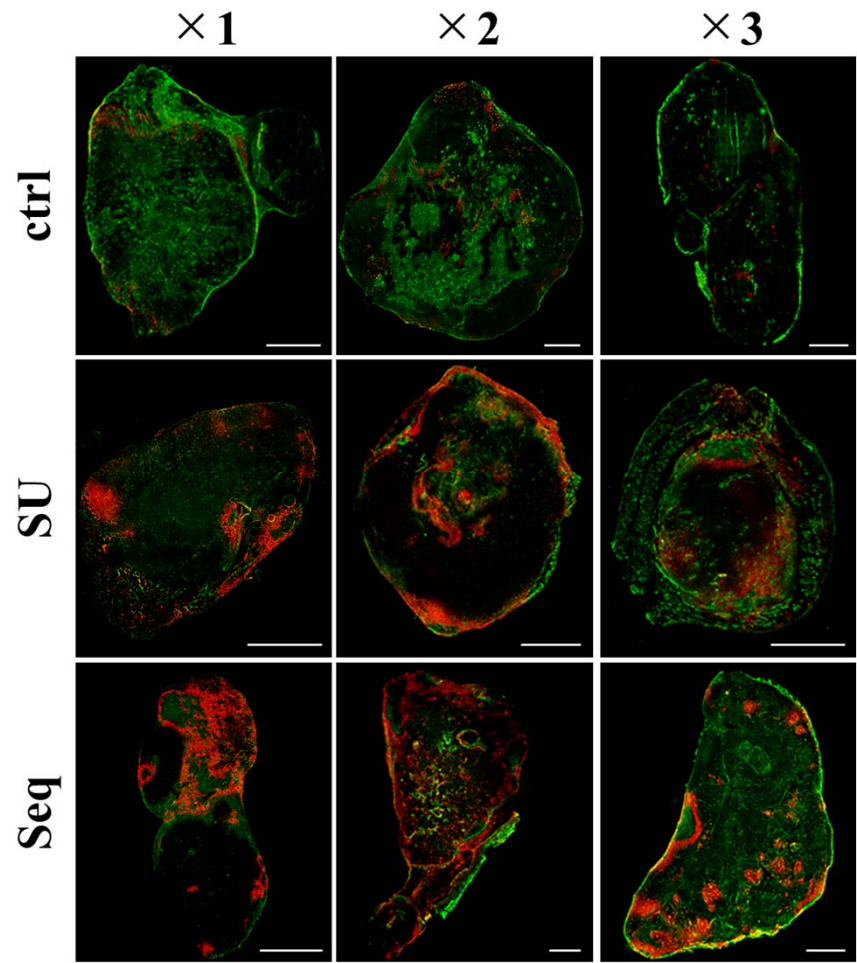


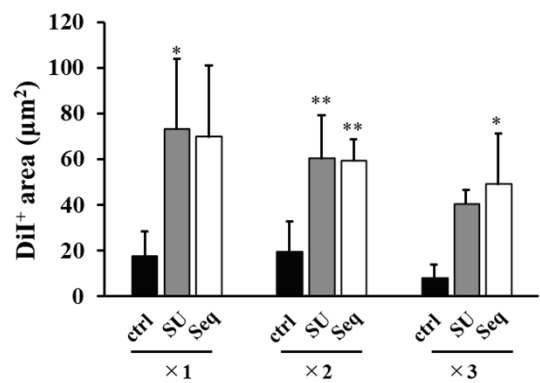
Figure 7 Maruyama et al.

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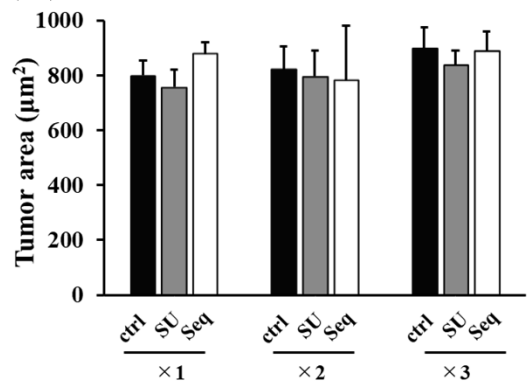


(B)

(i)



(ii)



(iii)

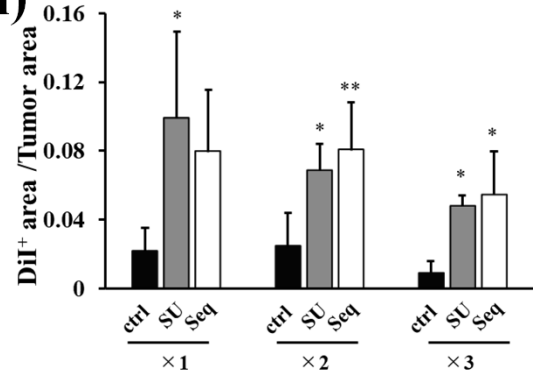
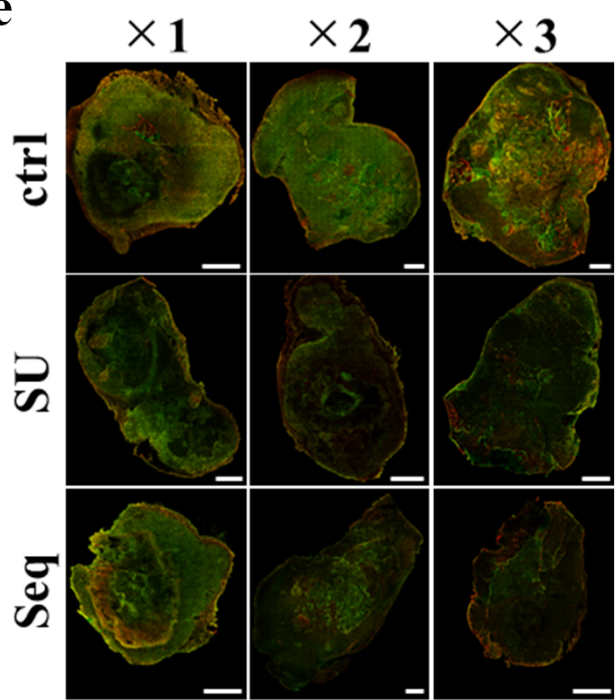
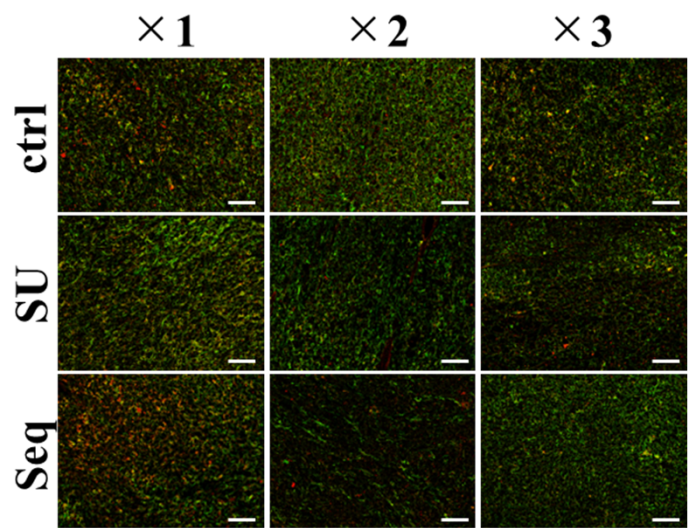


Figure 8 Maruyama et al.

(A) Whole



(B) Core



(C) Peripheral

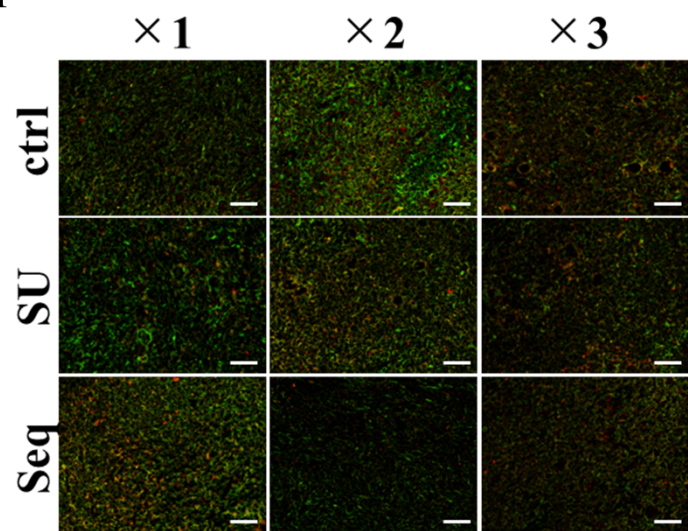


Figure 9 Maruyama et al.

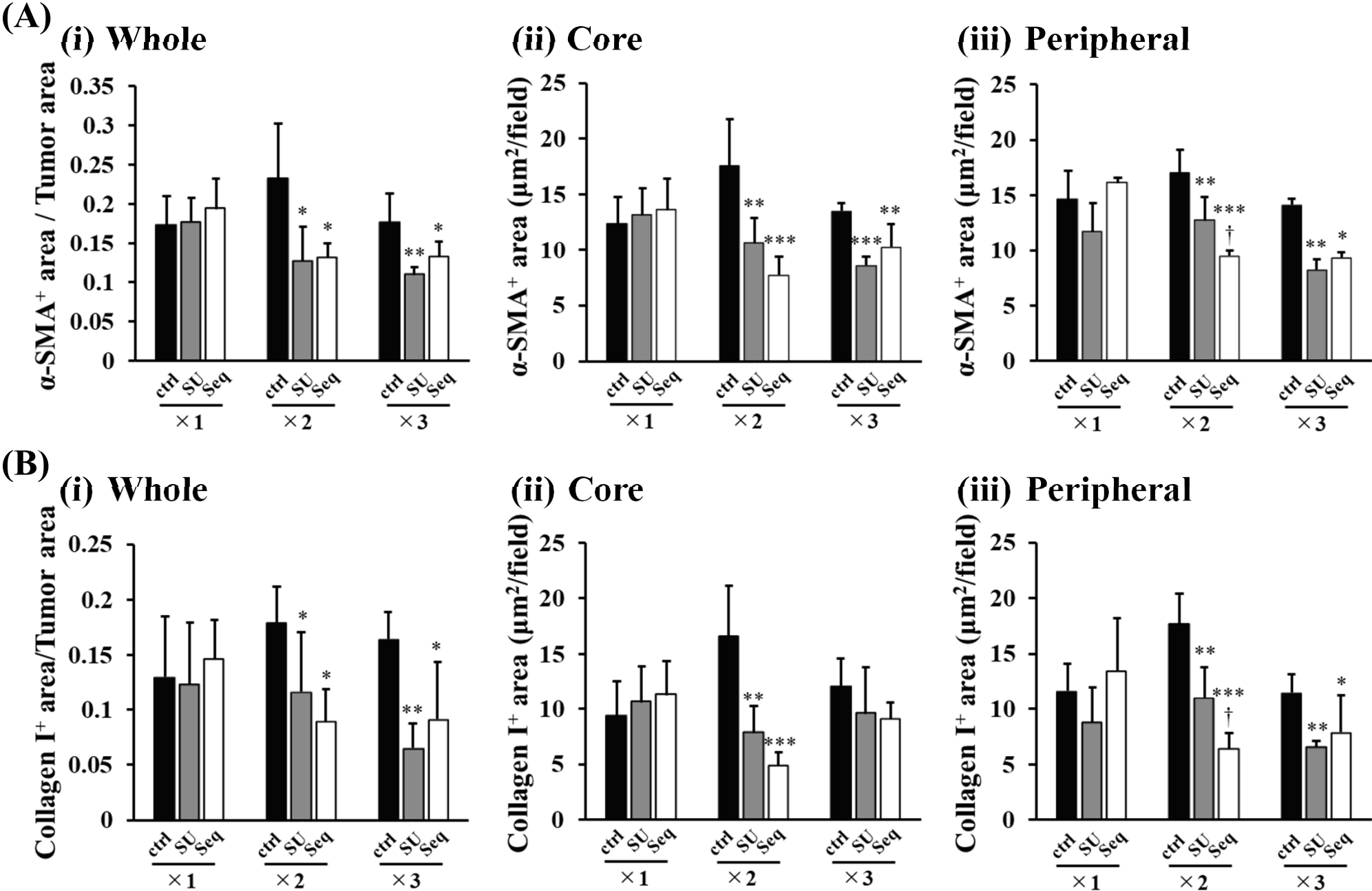
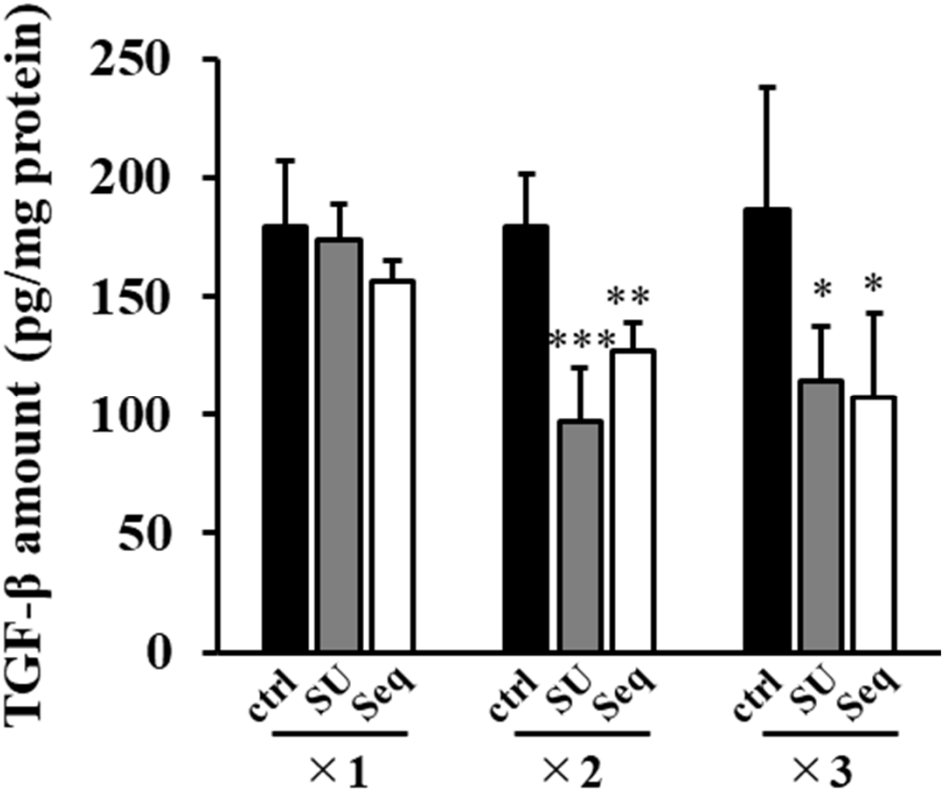
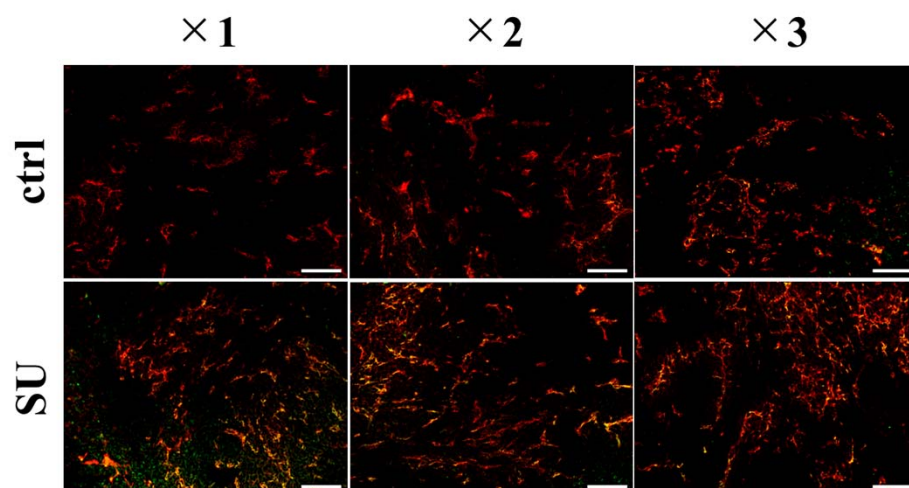


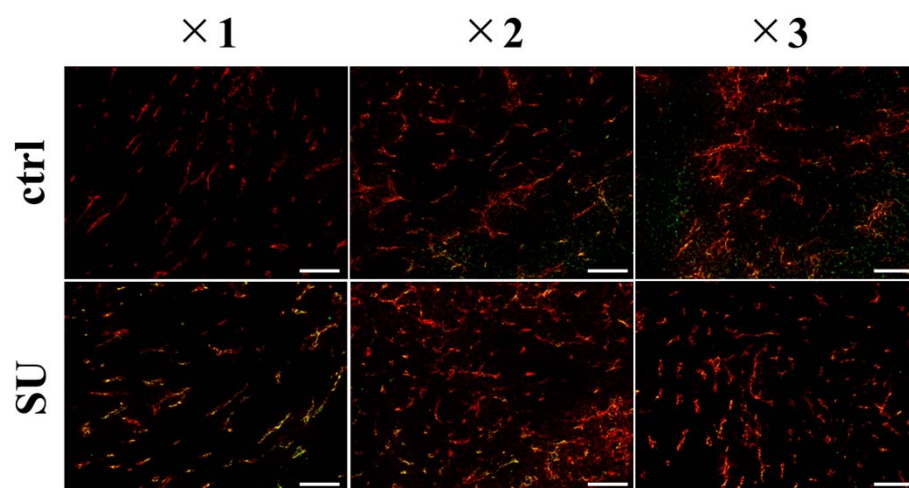
Figure 10 Maruyama et al.



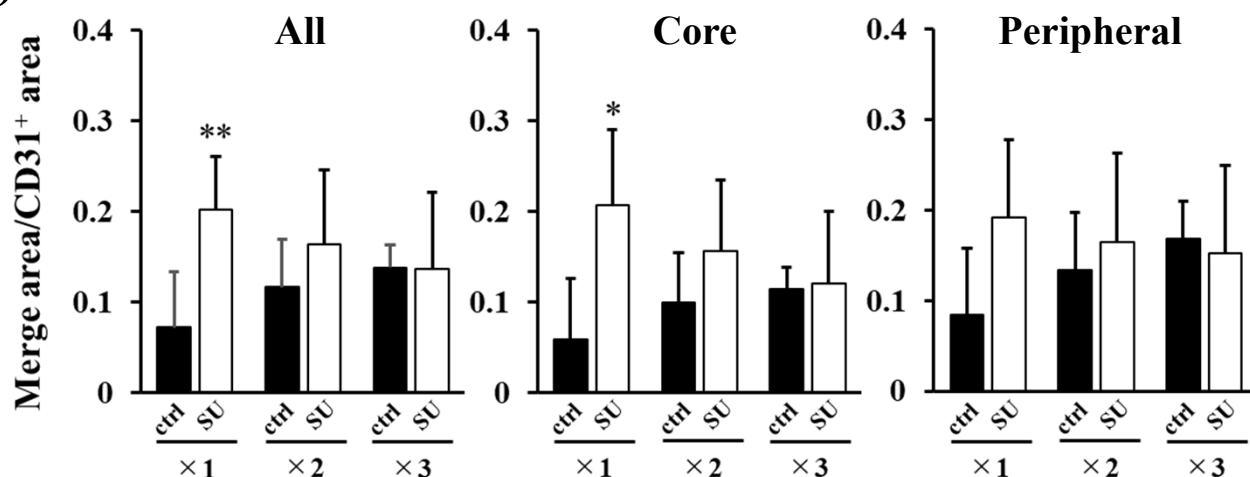
(A) Core



(B) Peripheral



(C)



Immunofluorescence images for VE-cadherin and vascular endothelial cells in 4T1 tumor tissues after repeated administration of PE-SU5416.

Tumor sections, prepared after one ($\times 1$), two ($\times 2$), and three ($\times 3$) administrations of PE-SU5416 (SU), were immunostained for VE-cadherin (green) and endothelial cells (CD31, red). Ctrl means an untreated control. Representative merged images for each group in the core (A) and peripheral (B) regions were shown. Scale bars, 100 μm . (C) Quantitative analysis of the ratio of VE-cadherin⁺CD31⁺ merged area/CD31⁺ area in all, the core, and peripheral regions of each group. “All” indicates the average of the core and peripheral regions. Results are expressed as the mean with S.D. bars (n=5).

**p< 0.01, *p< 0.05 compared with each ctrl group.