signals were localized predominantly in the capillaries and arterioles. FITC signals were also detected in myocytes at this time point. These data suggest that NP solution might distribute to intra- and extracellular spaces of ischemic skeletal muscle tissues immediately after intramuscular injection of NPs, and then the NP was uptaken by cells in injected muscles (endothelial cells, smooth muscle cells, myocytes, etc) or retained in extracellular spaces at this early time point.

On days 7 and 14, FITC signals remained localized predominantly in capillaries and arterioles (Figure 1B). Immunofluorescent staining revealed FITC signals localized mainly in endothelial cells positive for CD31, a marker of angiogenesis, in FITC-NPs injected ischemic muscle 14 days postischemia (supplemental Figure I). In contrast, no FITC signals were observed in myocytes. FITC signals were not detected in contralateral nonischemic hindlimb or in remote organs (liver, spleen, kidney, and heart) at any time point (data not shown).

Cellular Delivery of NPs Into Vascular Endothelial Cells Versus Skeletal Myocytes In Vitro

Cellular uptake of NPs was examined in HUVECs and SkMCs after incubation with FITC-NPs for 1 hour. The number of FITC-positive cells was greater among HUVECs than among SkMCs (supplemental Figure IIA). An inhibitor of clathrin-mediated endocytosis, chlorpromazine (CPZ), did not affect the magnitude of cellular FITC signals in SkMCs, but reduced the magnitude in HUVECs (supplemental Figure IIB). Long-term cell culture after 1-hour incubation with FITC-NPs revealed cellular FITC signals in HUVECs on days 3 and 7 postincubation (supplemental Figure IIC). In contrast, no FITC signal was detected in SkMC (data not shown).

Effects of Statin-NP on Ischemia-Induced Neovascularization

Treatment with statin-NP that contains pitavastatin at 0.4 mg/kg, but not with FITC-NP or statin only, significantly increased blood flow recovery on days 7 and 14 (Figure 2A and 2B). The beneficial effects of statin-NP were not associated with significant changes in serum biochemical markers (supplemental Table I), but angiogenesis and arteriogenesis were significantly increased (Figure 2C). Examination of hematoxylin-eosin-stained sections revealed no abnormal histopathologic findings (inflammation and fibrosis) among the 4 groups (data not shown). There was no significant difference in muscle fiber density among the 4 groups (data not shown).

Single intramuscular injection of nonnanoparticulated soluble pitavastatin at doses of 4 and 20 mg/kg exerted no effect on blood perfusion after hindlimb ischemia (supplemental Figure IIIA). Oral daily administration of pitavastatin at 0.4 mg/kg did not increase blood flow recovery, but pitavastatin at 1.0 and 10 mg/kg significantly increased blood flow recovery on day 14 (supplemental Figure IV).

Systemic daily administration of statins is reported to increase EPC mobilization,[18,26] but the EPC number in the circulating blood was not increased in the present study (supplemental Figure IIIB and IIIC). No therapeutic effects of

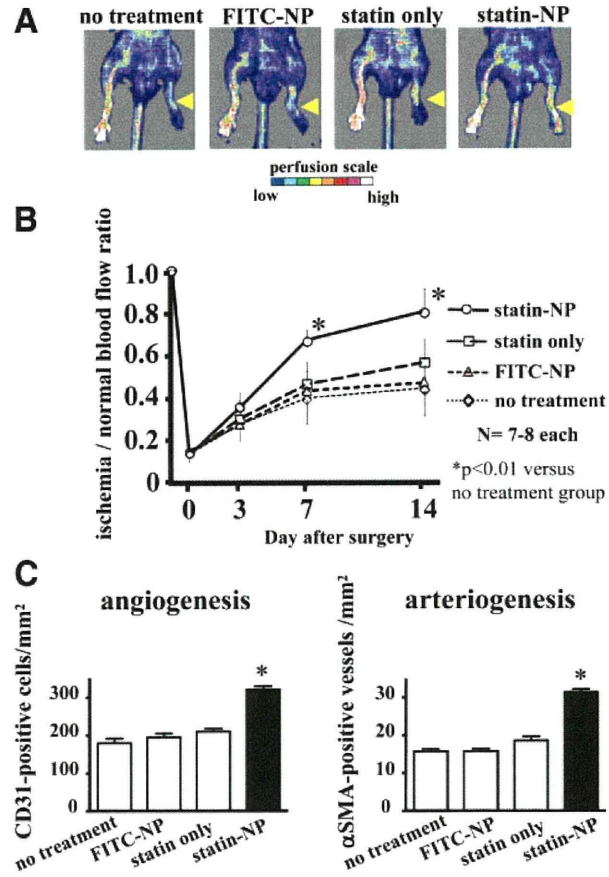

Figure 2. A, Representative laser Doppler perfusion imaging at 14 days postischemia. Arrow indicates ischemic limb. B, Quantification of blood flow recovery. C, Quantitative analysis of angiogenesis and arteriogenesis. n=4 each. *$P<0.01$ vs no treatment group.

statin-NP were observed in wild-type mice administered L-NAME or in eNOS$^{-/-}$ mice (Figure 3A), suggesting that eNOS-related signals are involved in the mechanism of statin-induced enhancement of ischemia-induced neovascularization (supplemental Figure V). Treatment with statin-NP increased both phosphorylated eNOS and serine-threonine specific protein kinase (Akt) in ischemic muscles compared with nonischemic control and nontreated ischemic muscles at 7 days of treatment (Figure 3B). Immunohistochemistry revealed that the increased eNOS and Akt activities were localized mainly in microvascular endothelial cells (supplemental Figure VI).

Effect of Statin-NP on Endogenous Angiogenic Growth Factor Expression

Immunohistochemistry was performed to examine the cellular localization of angiogenic growth factors in control and statin-NP groups. On day 3, immunostaining for both vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) was observed in skeletal myocytes and blood vessels (supplemental Figure VII). On days 7 and 14, the immunostaining intensity markedly decreased in skeletal myocytes and blood vessels in the control group. In contrast, positive immunostaining was observed in endothelial cells of

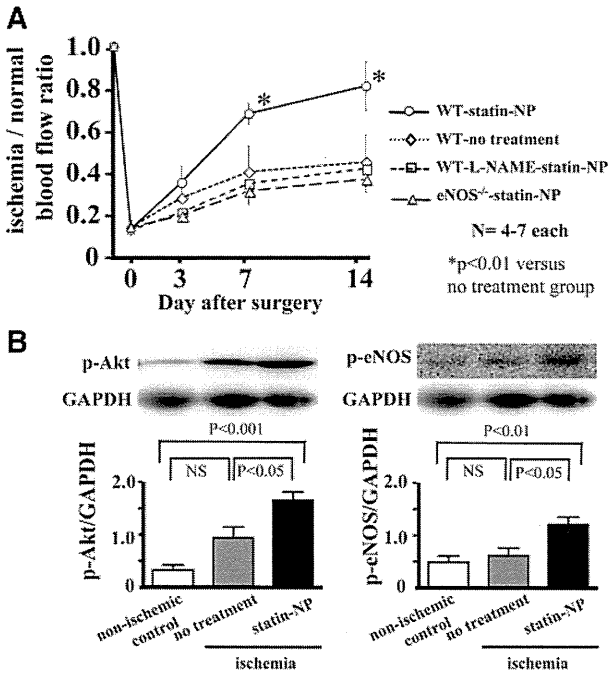

Figure 3. A, Quantification of blood flow recovery in wild-type (WT) mice with or without administration of L-NAME, a NOS inhibitor, and in eNOS$^{-/-}$ mice. B, Western blot analysis of phosphorylated Akt and eNOS in ischemic and nonischemic muscles 7 days after ischemia. n=6 each. NS=not significant.

capillaries and arterioles in the statin-NP group on days 7 and 14. Western blot analysis revealed greater protein expression of VEGF, FGF-2, and monocyte chemotactic protein-1 (MCP-1) in ischemic muscle in the statin-NP group than in the no treatment group 7 days after hindlimb ischemia (Figure 4). Interestingly, the increased expression of such angiogenic growth factors by treatment with statin-NP was blunted in mice administered chronically with L-NAME.

Effects of Statin-NP on Angiogenic Capacity of Human Endothelial Cells In Vitro

Cotreatment with statin or statin-NP increased angiogenic activity in HUVECs. The angiogenic activity of statin-NP was greater than that of 10 nmol/L statin only (supplemental Figure VIIIA). Pretreatment with statin only (24-hour incubation of HUVECs with statin) had no angiogenic effects at any dose. In contrast, pretreatment with statin-NP induced significant angiogenic effects at 1 and 10 nmol/L compared with the no-treatment control group (supplemental Figure VIIIB).

Serum and Tissue Concentrations of Statin

Tissue concentrations of pitavastatin were greater in skeletal muscles injected with statin-NPs than in those injected with statin 6 and 24 hours after intramuscular administration, whereas serum levels of pitavastatin were comparable between the 2 groups (supplemental Table II). The drug was not detected in serum 1 and 3 days after injection.

Discussion

The application of nanotechnology-based drug delivery is expected to have a major impact on the development of

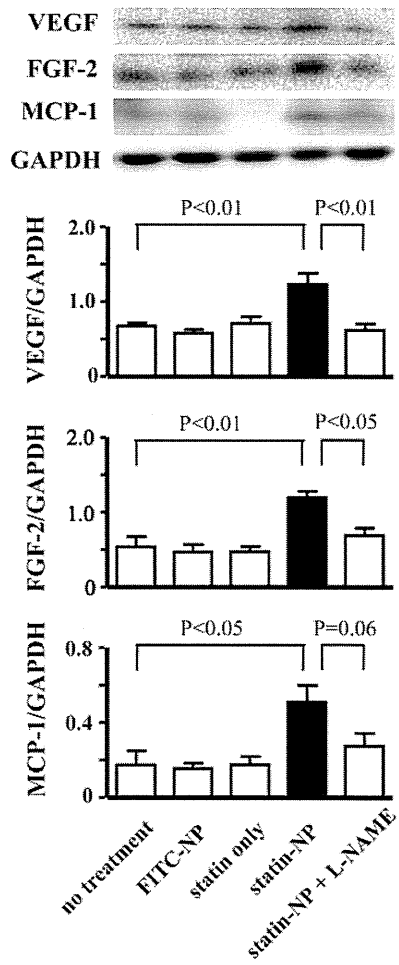

Figure 4. Effects of statin-NP on expression of VEGF, FGF-2, and MCP-1 in ischemic muscle. Densitometric analysis of protein expression in ischemic muscles 7 days after ischemia. Quantitative evaluation was expressed as a ratio of VEGF, FGF-2, and MCP-1 to GAPDH. n=6 each.

innovative medicines. In the present study, selective NP-mediated delivery of statin to vascular endothelial cells increased neovascularization and improved tissue perfusion in a murine model of hindlimb ischemia, indicating that this novel cell-selective delivery system is feasible for therapeutic neovascularization.

The most novel finding of this study is that FITC signals were localized mainly in the vascular endothelium 7 and 14 days after injection of FITC-NP into ischemic skeletal muscles in vivo. Several factors might be involved in mechanisms of the cell-selective delivery of the NP at later time points. First, increased endocytosis of NP in the endothelium may be involved, which is based on our present experiments with CPZ, an inhibitor of clathrin-mediated endocytosis. In addition, 1-hour incubation with FITC-NP resulted in long-term and stable retention of NP in the human endothelial cells, but not in skeletal myocytes in vitro. Second, decreased exocytosis of the endothelium in the presence of ischemia might also be involved. Third, after cellular delivery of NP via endocyotosis, rapid escape of the NP from the endosomal compartment to the cytoplasmic compartment may lead to

sustained intracellular drug delivery and good efficacy. The NP is likely retained in the cytoplasm where release of the encapsulated drug occurs slowly in conjunction with the hydrolysis of PLGA.[15] Overall, the nanotechnology platform for cell-selective delivery to the vascular endothelium using NP may be useful as an innovative strategy for therapeutic neovascularization and other intractable diseases.

Another important feature of this study is that a single administration of statin-NP containing pitavastatin (0.4 mg/kg) into vascular endothelial cells effectively increased therapeutic neovascularization with no serious side effect in murine model of hindlimb ischemia. Sata et al[24] reported that systemic daily administration of pitavastatin (1 mg/kg per day × 49 days=49 mg/kg) has significant therapeutic effects in mice with hindlimb ischemia. In the present study, we confirmed the study of Sata et al[24] by showing that oral daily administration of pitavastatin for 14 days (1 and 10 mg/kg per day × 14=14 and 140 mg/kg, respectively) had significant therapeutic effects, as did statin-NP (0.4 mg/kg). Therefore, our NP-mediated delivery system seems to be as effective at an approximately 100-times lower dose than the cumulative systemic dose. Furthermore, measurement of the tissue and serum concentrations of pitavastatin confirmed the effective local retention of statin-NPs in ischemic skeletal muscles in vivo. NP-mediated delivery of pitavastatin accelerated angiogenic activity of human endothelial cells in vitro. Therefore, it is possible that after NP-mediated endothelial delivery, pitavastatin was slowly released from the NPs into the cytoplasm along with PLGA hydrolysis, resulting in significant therapeutic effects.

Clearly, the therapeutic neovascularization induced by statin-NPs resulted from the pleiotropic effects, because pitavastatin-NPs had no effect on serum lipid levels. Our experiments with mice treated with a NOS inhibitor and $eNOS^{-/-}$ mice support the essential role of the eNOS pathway in the mechanism underlying the therapeutic effects of NP-mediated cell-selective delivery of statin. Consistent with the results of other investigators,[18,20,21,26] we demonstrated that pitavastatin-NP increased the activity of vascular eNOS and PI3K/Akt (as shown in supplemental Figure V) in association with an increased expression of endogenous multiple angiogenic growth factors that are involved in angiogenesis (VEGF) and arteriogenesis (FGF-2, MCP-1).[27] These therapeutic effects afforded by the NP-mediated cell-selective delivery of statin were not associated with a further increase in circulating EPC. Intramuscular injection of soluble pitavastatin alone at high doses (4 and 20 mg/kg) has no therapeutic effect, suggesting a specific advantage of endothelial cell selective delivery of pitavastatin by the PLGA NP formulation. These findings suggest that pitavastatin-NP acted locally on ischemic vascular endothelium to induce therapeutic neovascularization and are consistent with the notion that NP-mediated endothelial cell-selective delivery of statin produces a well-harmonized integrative system to form functionally mature collaterals via controlled expression of endogenous multiple angiogenic growth factors and signals, allowing for a more effective model for an integrative approach to therapeutic neovascularization.

There is a major limitation to the present study. First, we examined only a single dose of statin-NPs. It is difficult to obtain a dose-response relationship of this NP system in small animals. For translation of our present findings into clinical medicine, further studies are needed to define the dose-response relation in large animal models. This point is important because statins are reported to exert a double-edged role in angiogenesis signaling.[28] Although such antiangiogenic effects of statins at high dose did not occur in a murine model,[24] this must be examined in large animal models. Second, we only examined the therapeutic effects of a single intramuscular injection of statin-NP. Whether repetitive delivery of statin-NP at an optimal dose over time produces greater therapeutic effects remains to be investigated.

In conclusion, this platform nanotechnology of vascular endothelial cell-selective delivery of statin is a promising strategy toward more effective and integrative nanomedicine in patients with severe organ ischemia, and represents a significant advance in therapeutic neovascularization over current approaches. The nanotechnology platform may be developed further as an "integrative" approach for therapeutic neovascularization, and extended to target other molecular signals specific to vascular endothelial cells.

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Disclosures

Dr Egashira holds a patent on the results reported in the present study. The remaining authors report no conflicts.

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Supplement Data

Therapeutic Neovascularization by Nanotechnology-Mediated Cell-Selective Delivery of Pitavastatin into the Vascular Endothelium

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Supplementary Table 1.

Serum biochemical profiles 3, 7 and 14 days after hindlimb ischemia

	No treatment	FITC-NP	Statin only	Statin-NP	*P* value
Creatine Phosphokinase (IU/L)					
day 3	105±16	144±25	138±26	141±29	0.66
day 7	75±12	100±14	83±16	65±10	0.35
day 14	76±13	56±7	74±3	52±17	0.39
Myoglobin (ng/ml)					
day 3	< 10	< 10	< 10	< 10	-
day 7	< 10	< 10	< 10	< 10	-
day 14	< 10	< 10	< 10	< 10	-
AST (IU/L)					
day 3	63±10	54±24	64±14	60±16	0.97
day 7	34±1	37±6	47±8	32±7	0.37
day 14	35±7	29±2	38±5	35±3	0.57
ALT (IU/L)					
day 3	27±5	24±1	22±3	27±8	0.86
day 7	13±1	23±10	18±3	15±4	0.64
day 14	19±1	17±0	24±5	22±3	0.35
BUN (mg/dl)					
day 3	26±2.5	29±1.5	30±0.3	29±3.6	0.75
day 7	28±1	23±5	22±1	22±1	0.44
day 14	33±2	34±2	35±1	37±1	0.36
Creatinine (mg/dl)					
day 3	0.11±0.01	0.10±0.01	0.14±0.01	0.14±0.02	0.11
day 7	0.10±0.01	0.10±0.01	0.11±0.01	0.10±0.01	0.69
day 14	0.12±0.01	0.11±0.02	0.10±0.01	0.10±0.01	0.50
Total cholesterol (mg/dl)					
day 3	95±3	102±1	90±2	105±12	0.40
day 7	87±4	92±11	81±4	76±3	0.39
day 14	76±2	84±5	87±5	80±2	0.25
LDL cholesterol (mg/dl)					
day 3	24±2	22±2	24±3	27±5	0.72
day 7	12±2	21±9	15±2	13±2	0.56
day 14	5±1	5±0	8±1	7±2	0.33

Data are mean±SEM (n=3 each)

Supplementary Table 2.

Tissue and serum pitavastatin concentrations after intramuscular injection of statin

	time after injection		
	6 hours	1 day	3 day
Pitavastatin at 0.4 mg/kg			
muscle (ng/g tissue)	305 ± 80	81 ± 60	4 ± 3
serum (ng/ml)	3 ± 0.3	ND	ND
Statin-NP containing 0.4 mg/kg of pitavastatin			
muscle (ng/g tissue)	2088 ± 412*	692 ± 288*	9 ± 5
serum (ng/ml)	5 ± 0.7	ND	ND

Data are mean±SEM (n=8 to 9 each). ND: not detected. *P<0.01 versus intramuscular pitavastatin.

Supplementary Figure Legends

Supplementary Figure I. Immunofluorescent staining of cross-sections from ischemic muscle 14 days after FITC-NP injection stained with an endothelial marker, CD31 (red). Inset left below is non-injected control muscle. Scale bars: 100 μm.

Supplementary Figure II. Cell-selective delivery of FITC-NP into vascular endothelial cells versus skeletal myocytes. A, Fluorescent micrographs of HUVEC and SkMC incubated with FITC-NP (0.1 mg/ml) for 1 hour and percentage of FITC-positive cells (n=5 each). Nuclei were counter stained with PI. Scale bars: 100 μm. B, Effects of chlorpromazine (CPZ) on cellular distribution of NP in HUVEC and SkMC. Quantitative analysis of magnitude of intracellular FITC fluorescence signals in 3 independent experiments are shown. *$p<0.05$, **$p<0.001$ versus control condition. C, Fluorescent micrographs of HUVEC immediately after, and 3 and 7 days after the 1 hour incubation with FITC-NPs. Inset left above is control HUVEC without FITC-NP. Nuclei were counter stained with PI. Scale bars: 100 μm.

Supplementary Figure III. A, Quantification of LDPI–derived blood flow recovery expressed as the ratio of ischemic to normal limb at 7 and 14 days. Mice were injected with pitavastatin at 4 and 20 mg/kg into the ischemic muscle immediately after induction of hindlimb ischemia. N = 5 to 6. NS=no significance. B, Representative scatter diagram of Sca-1/Flk-1-double positive EPCs in peripheral blood analyzed by flow cytometry 14 days after induction of hindlimb ischemia (circled region). C, Quantitative analysis of circulating EPCs expressed as percentage of Sca-1/Flk-1 double positive cells to total leukocytes 7 and 14 days after ischemia. N = 4 to 5 each. *$p<0.05$, **$p<0.001$ versus non-ischemic control group.

Supplementary Figure IV. Effects of oral daily administration of pitavastatin on ischemia-induced neovascularization. Quantification of laser Doppler perfusion imaging (LDPI)–derived blood flow recovery at 14 days. n=6 to 8. *$p<0.05$, **$p<0.01$ versus no treatment group.

Supplementary Figure V. Schematic illustration of the effects of statins on intracellular pathways. Mevalonate, the end product of the HMG-CoA, inhibits PI3K and the subsequent phosphorylation of Akt and eNOS. Blockade of HMG-CoA reductase with statins is expected to result in the increase of activity of Akt and eNOS.

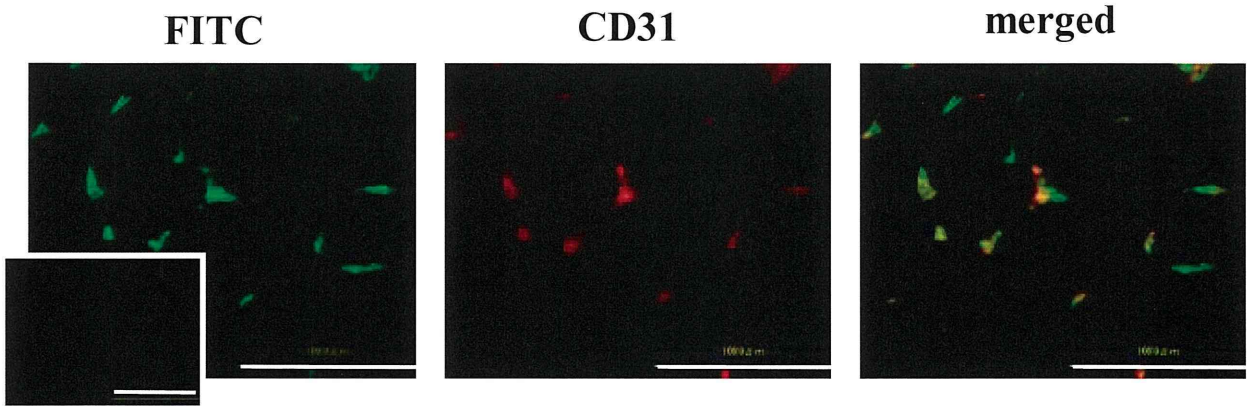
Supplementary Figure VI. Representative micrographs of ischemic muscle sections stained immunohistochemically with antibodies against phospho-Akt and phospho-eNOS at 14 days after surgery. Scale bars: 100 μm.

Supplementary Figure VII. Effects of statin-NP on the protein expression of VEGF (A) and FGF-2 (B) in ischemic muscle. Representative photographs of immunostaing of ischemic muscles 3, 7, and 14 days after hindlimb ischemia. VEGF or FGF-2 (green) is located not only within myocytes but within capillary or vascular endothelium (yellow) on day 7 and 14 in statin-NP group. Nuclei were counter stained with DAPI (blue). Scale bars: 100 μm.

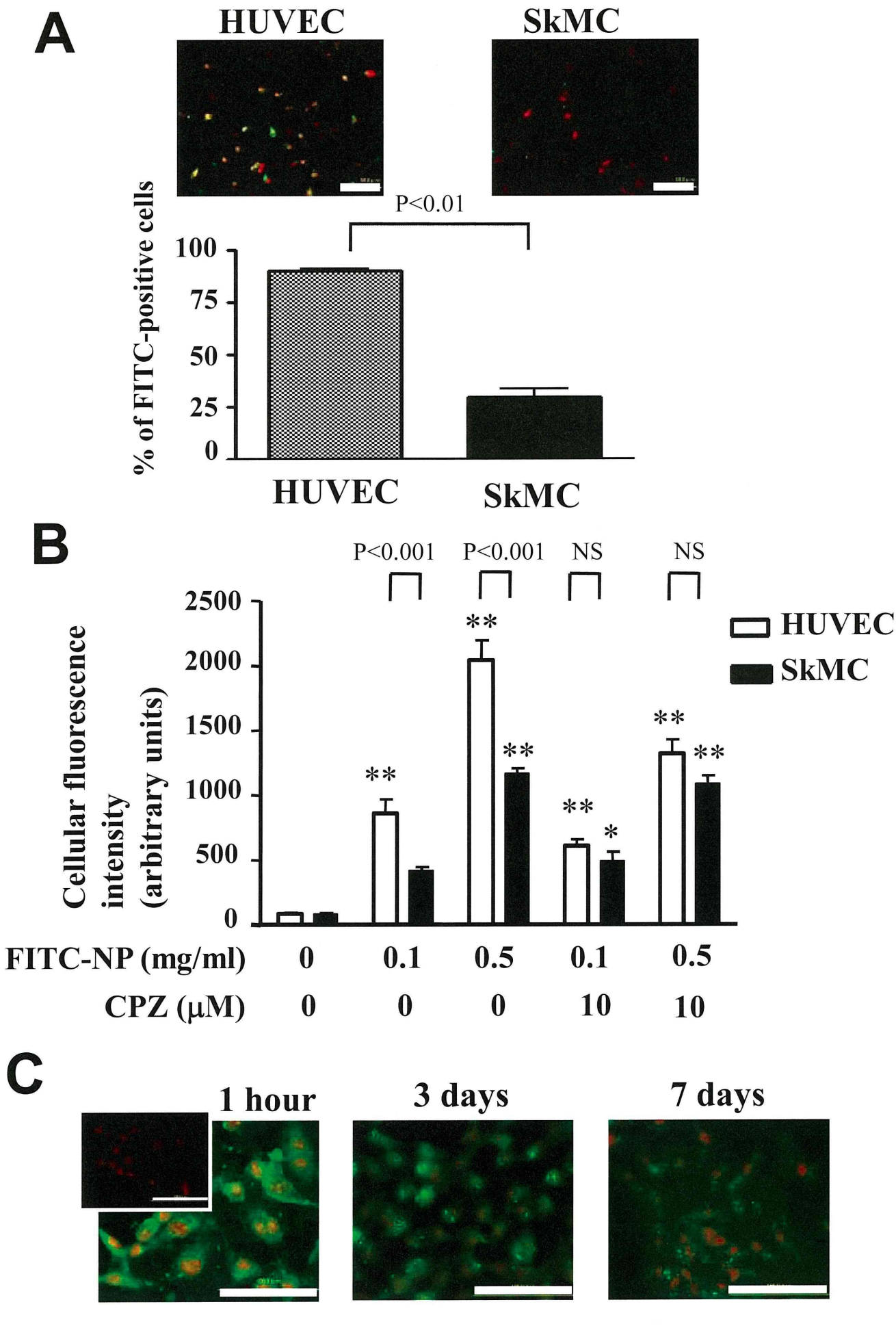
Supplementary Figure VIII. Effects of statin-NP on angiogenic capacity of human endothelial cells in vitro. A, Quantitative analysis of tube formation (tube length) of 4

independent experiments in co-treatment protocol. *$p<0.01$, **$p<0.001$ vs control. B, Quantitative analysis of tube formation (tube length) of 4 independent experiments in pre-treatment protocol. *$p<0.05$, **$p<0.001$ versus control.

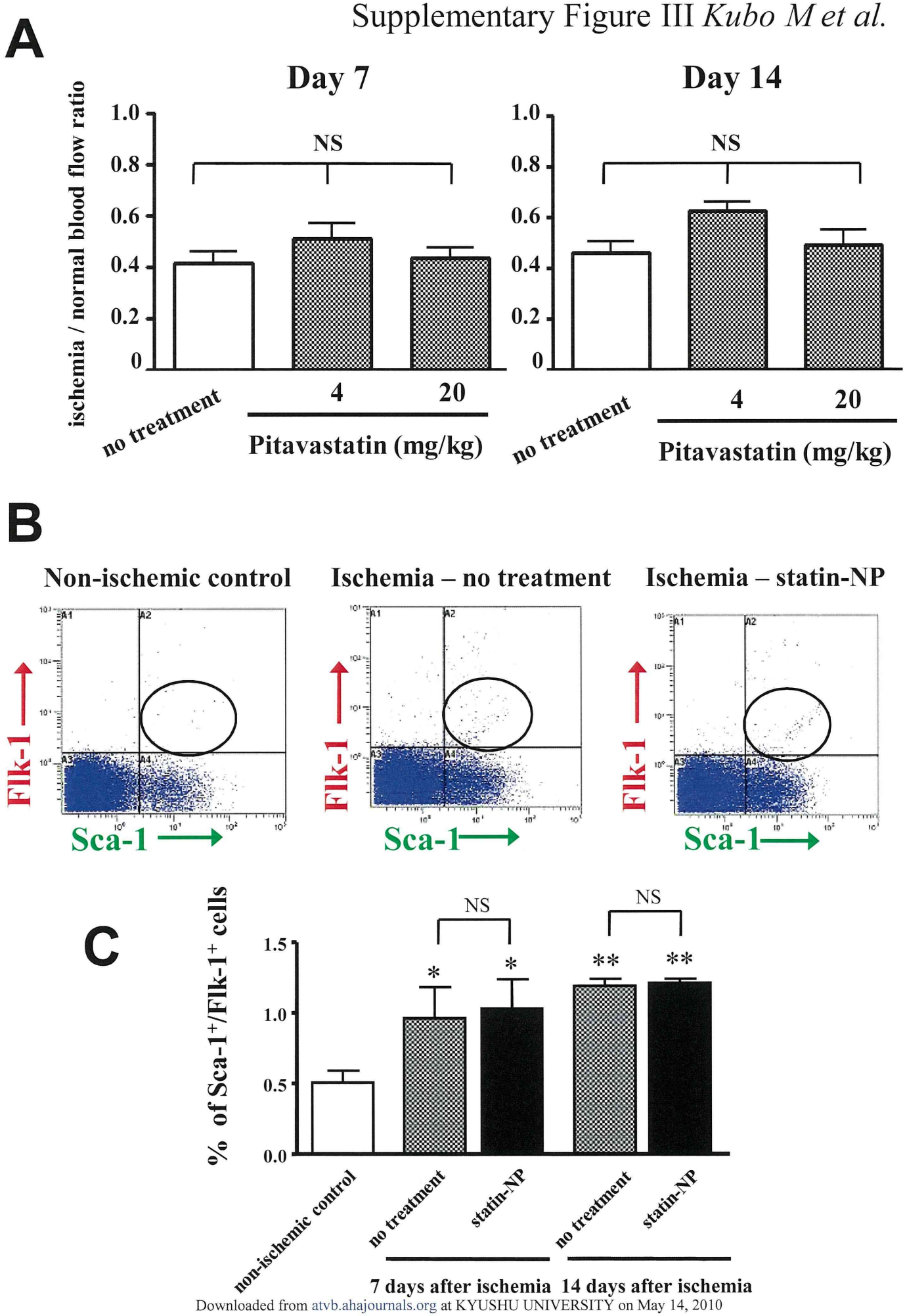
Supplementary Figure I *Kubo M et al.*



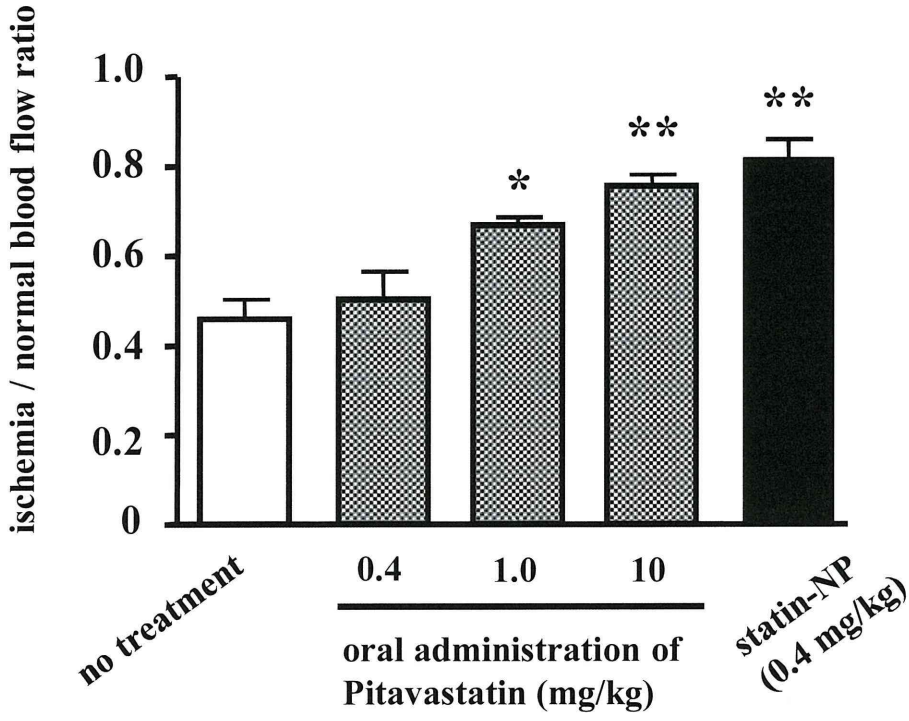
Supplementary Figure II *Kubo M et al.*



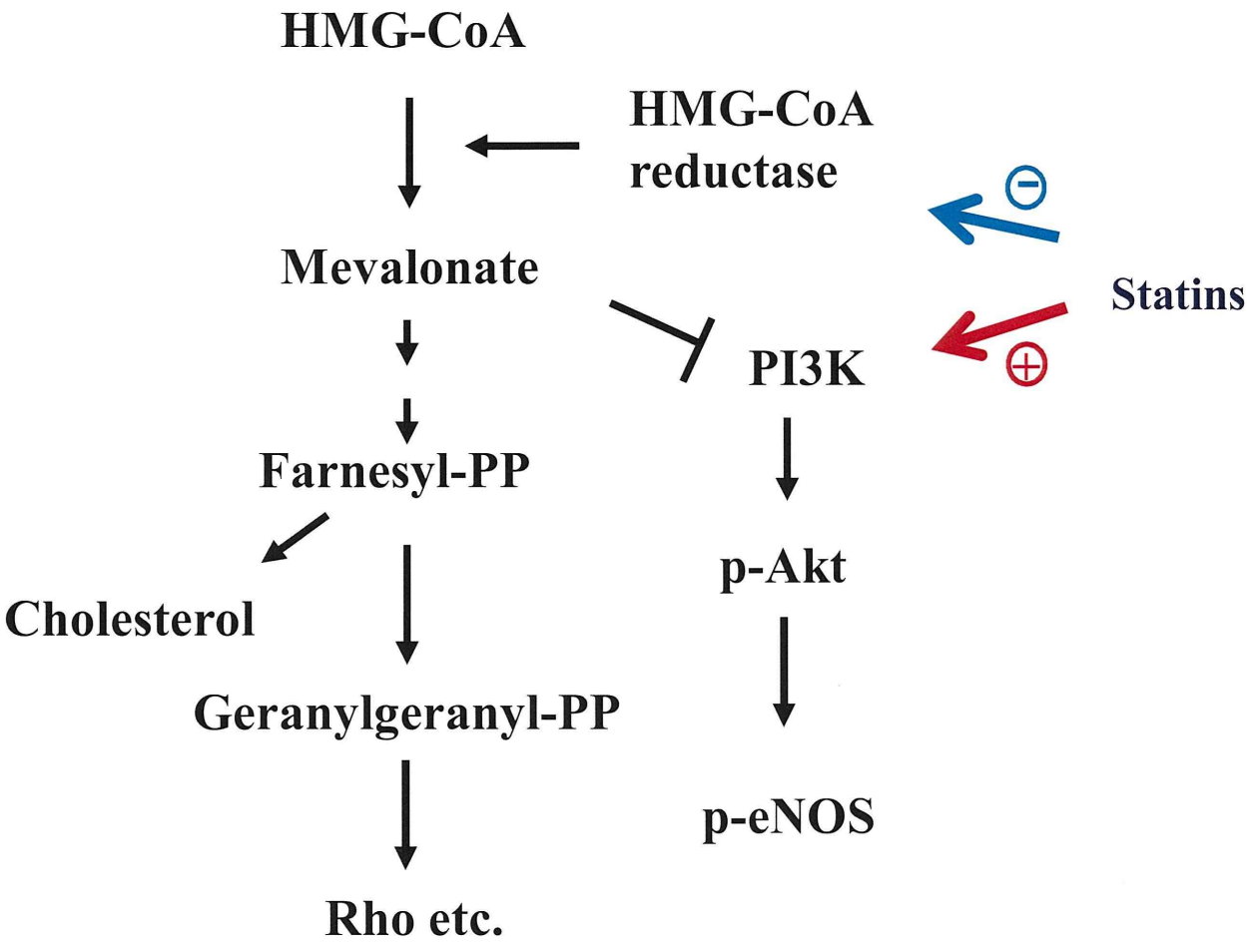
Supplementary Figure III *Kubo M et al.*



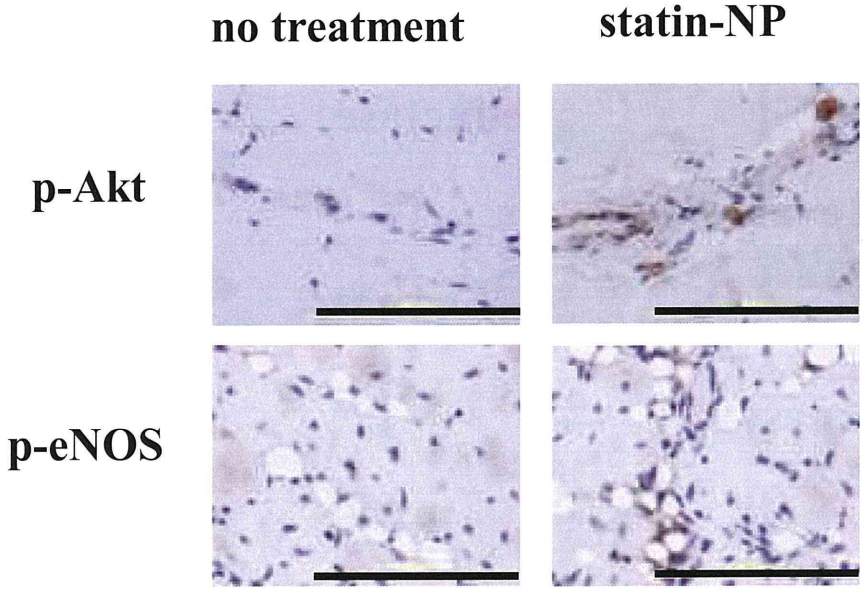
Supplementary Figure IV*Kubo M et al.*



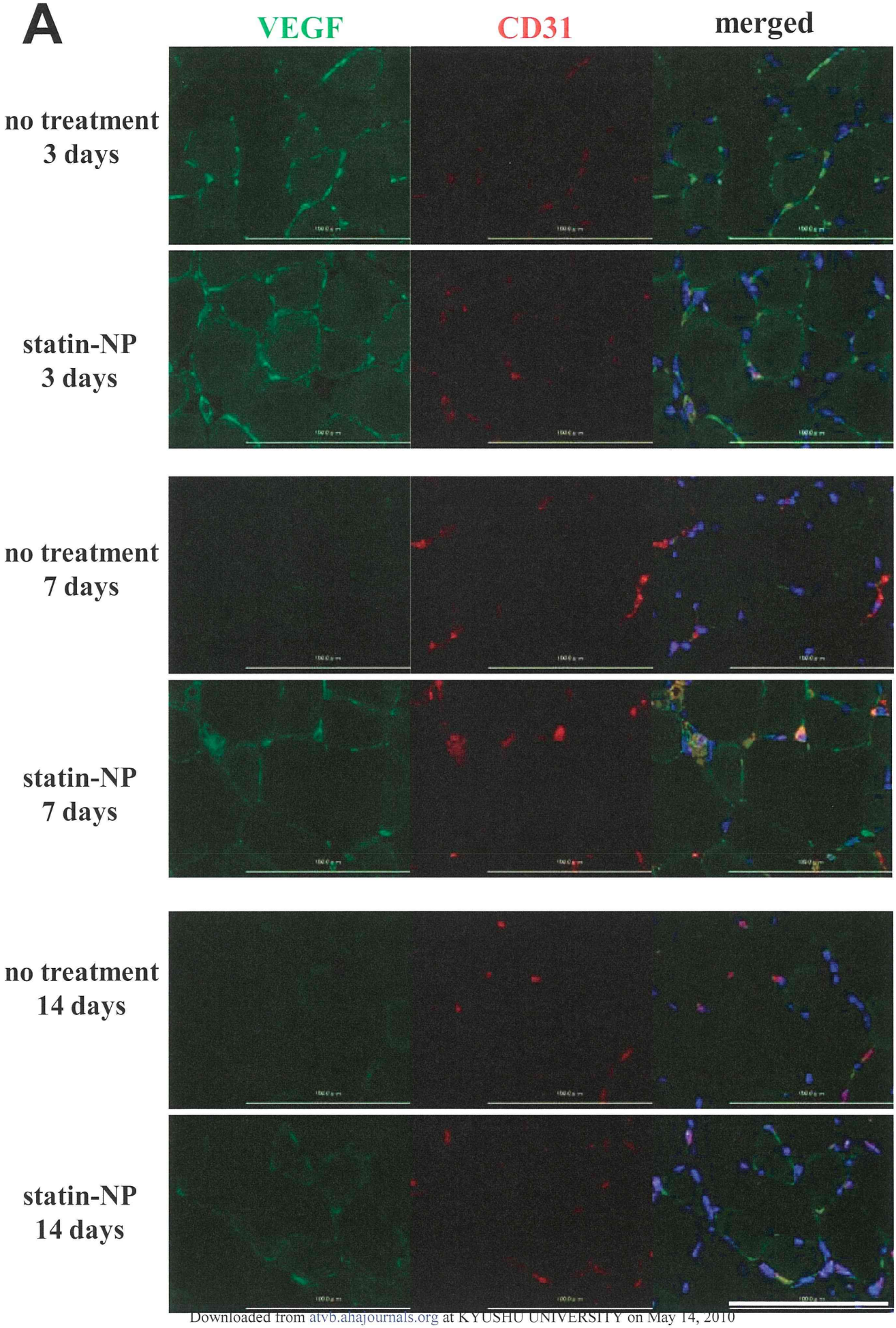
Supplementary Figure V *Kubo M et al.*



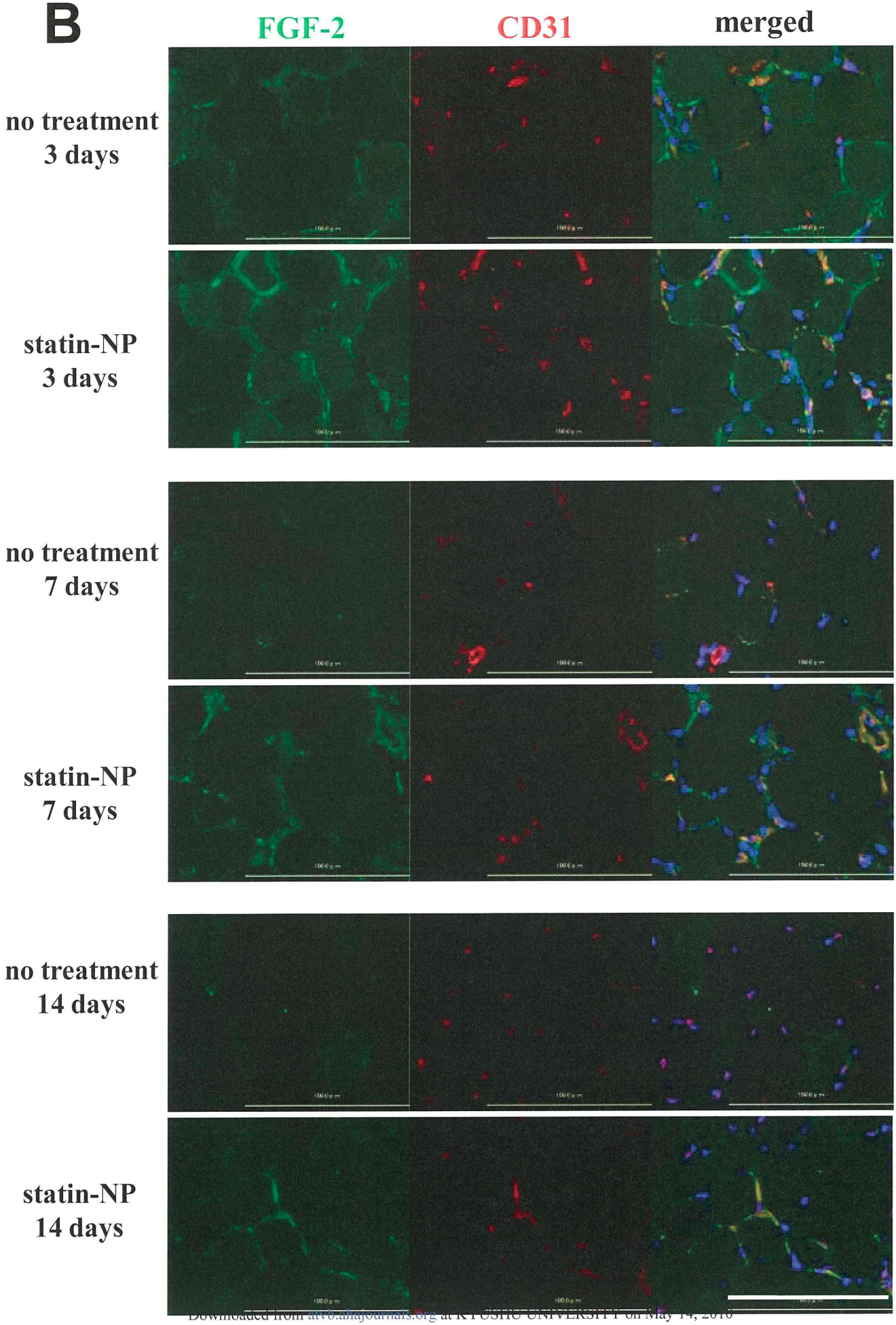
Supplementary Figure VI *Kubo M et al.*



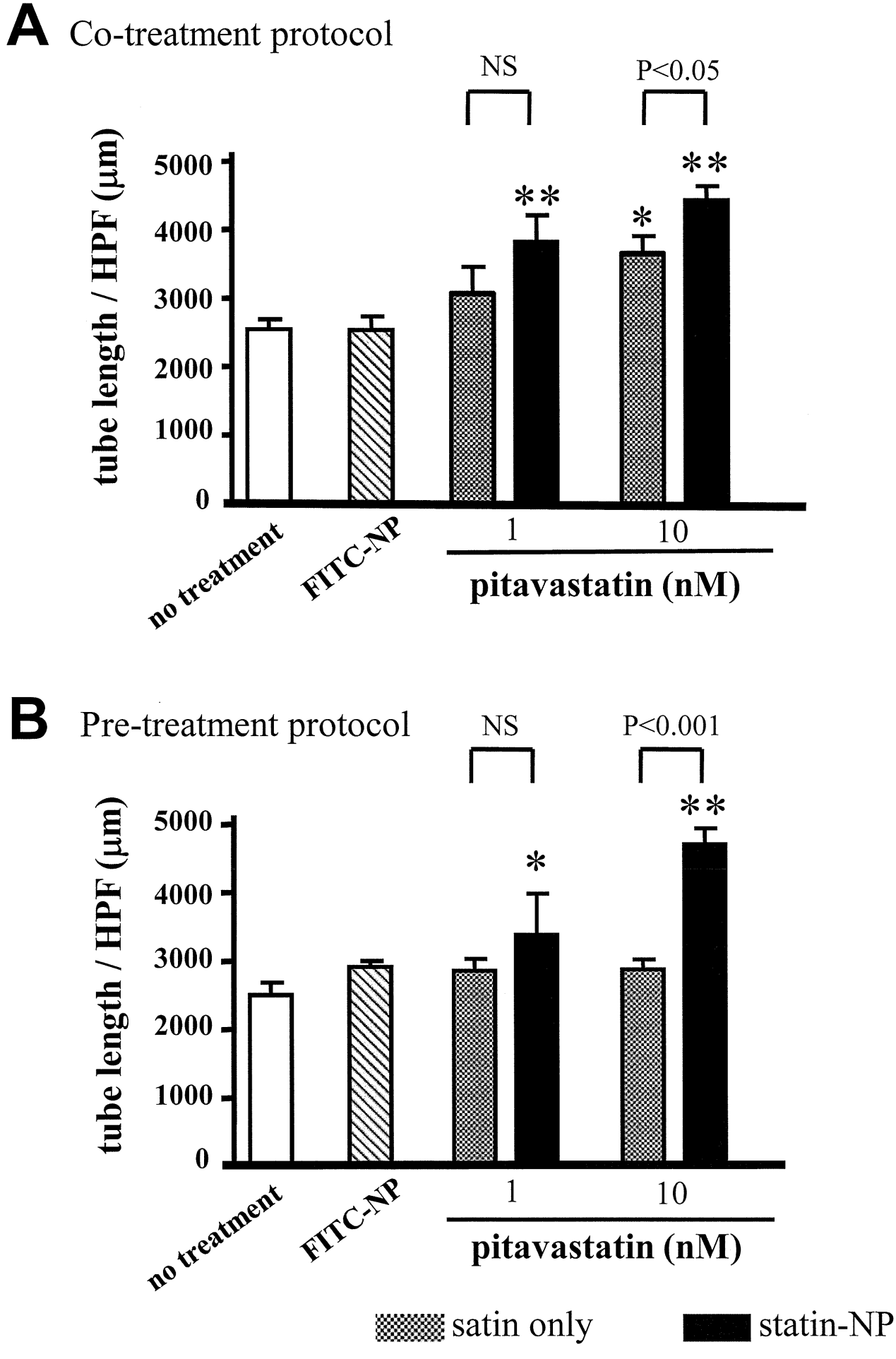
Supplementary Figure VII *Kubo M et al.*



Supplementary Figure VII *Kubo M et al.*



Supplementary Figure VIII *Kubo M et al.*



Supplement Material

Therapeutic Neovascularization by Nanotechnology-Mediated Cell-Selective Delivery of Pitavastatin into the Vascular Endothelium

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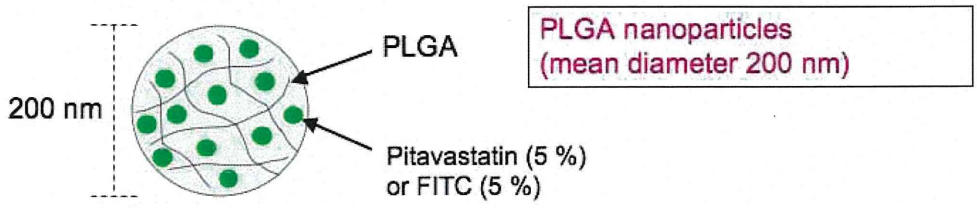
Materials and Methods

Preparation of PLGA NP

A lactide/glycolide copolymer (PLGA) with an average molecular weight of 20,000 and a copolymer ratio of lactide to glycolide of 75:25 (Wako Pure Chemical Industries, Osaka, Japan) was used as a wall material for the NP. According to manufacturer's instruction, a bioabsorption half-life of this product is 2 weeks in rat tissue.[1] Polyvinylalcohol (PVA-403; Kuraray, Osaka, Japan) was used as a dispersing agent. Fluorescein-isothiocyanate (FITC; Dojin Chemical, Tokyo, Japan) was used as a fluorescent marker of the NP.

We prepared bioabsorbable poly-lactide-glycolide copolymer (PLGA) nanoparticles (NP) by emulsion solvent diffusion method. The encapsulated agents are entrapped into the polymer matrix as shown below.



Advantages of PLGA NP-based drug delivery system (DDS) include:

- Matrix polymer (PLGA) is bioabsorbable.
- NP can incorporate water-soluble drugs/oligonucleotides/DNAs.
- NP can cross cell membrane via endocytosis (efficiency of cellular uptake: 90 % or more), and deliver the encapsulated agents into the cytoplasm.
- Incorporated drugs are slowly released from NP with hydrolysis of PLGA, which works intracellular DDS after intracellular uptake.

PLGA NP incorporated with FITC or pitavastatin (Kowa Pharmaceutical Co. Ltd., Tokyo, Japan) were prepared by a previously reported emulsion solvent diffusion method in purified water[2, 3]. PLGA were dissolved in a mixture of acetone and methanol. Then, FITC or pitavastatin were added into this solution. The resultant polymer-FITC or polymer-statin solution was emulsified in PVA solution under stirring at 400 rpm using the propeller-type agitator with three blades (Heidon 600G; Shinto Scientific, Japan). After agitating the system for 2 h under reduced pressure at 40 °C, the entire suspension was centrifuged (20,000×*g* for 20 min at -20 °C). After removing the supernatant, purified water was added to mix with the sediment. The wet mixture was then centrifuged again to remove the excess PVA and the unencapsulated reagent that could not adsorb on the surfaces of NP. After repeating this process, the resultant dispersion was freeze-dried under the same conditions. The FITC- and pitavastatin-loaded PLGA NP contained 5 % (w/v) FITC and 5 % (w/v) pitavastatin, respectively.

Particle size and surface charge measurements

Scanning electron microscopy picture of the PLGA NP indicates that the NP is prepared in the form of powder. The mean particle size was analyzed by light scattering