1 **The dynamics of revascularization after white matter infarction monitored in Flt1-tdsRed and**
2 **Flk1-GFP mice**
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20 endothelial cell
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1 **Abstract**

2 Subcortical white matter infarction causes ischemic demyelination and loss of brain functions, as the
3 result of disturbances of the blood flow. Although angiogenesis is one of the recovery processes after
4 cerebral infarction, the dynamics of revascularization after white matter infarction still remains
5 unclear. We induced white matter infarction in the internal capsule of Flk1-GFP::Flt1-tdsRed double
6 transgenic mice by injection of endothelin-1 (ET-1), a vasoconstrictor peptide, together with N(G)-
7 nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, and followed the changes
8 in Flk1 and Flt1 expression in the vascular system in the infarct area. Reduction of Flt1-tdsRed-
9 positive blood vessels 1 day after the injection and increase of Flk1-GFP-strongly-positive blood
10 vessels 3 days after the injection were apparent. PDGFRβ-strongly-positive (PDGFRβ+) cells
11 appeared in the infarct area 3 days after the injection and increased their number thereafter. Three
12 days after the injection, most of these cells were in close contact with Flk1-GFP-positive endothelial
13 cells, indicating these cells are bona fide pericytes. Seven days after the injection, the number of
14 PDGFRβ+ cells increased dramatically, and the vast majority of these cells were not in close contact
15 with Flk1-GFP-positive endothelial cells. Taken together, our results suggest revascularization begins
16 early after the ischemic insult, and the emerging pericytes first ensheath blood vessels and then
17 produce fibroblast-like cells not directly associated with blood vessels.

18

1. Introduction

Subcortical white matter infarction causes ischemic demyelination, loss of brain functions, and substantial handicap as a result of disturbances of the blood flow [2]. Although early infarction could be asymptomatic, it is progressive, resulting in cognitive and motor dysfunction. It is the second leading cause of dementia [14]. Immediately following the interruption of the blood flow to brain tissue, the progressive decrease of the oxygen concentration initiates the death of cells in the ischemic lesion, unless recovery of the normal blood flow occurs within a short time [7]. Capillary endothelial cells, too, die under hypoxic condition, because of their high rate of oxidative metabolism [9]. Endothelial cells are also very vulnerable to the reactive oxygen species produced in the reperfusion stage of the ischemic insult [20]. The dynamics of vascularization in detail after the white matter infarction, however, is still unclear. This is one of the reasons why the fundamental therapeutic strategies for the white matter infarction have not been established. Another reason is the paucity of researches using the white matter infarction animal models compared with the large artery occlusion models, as the volume of white matter in rodents is much smaller than that in primates [18].

The present study aimed to examine the dynamics of revascularization after the white matter infarction in mice. We induced white matter infarction by stereotactic injection of endothelin-1 (ET-1), a potent vasoconstrictor, together with N(G)-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, into the internal capsule of mice. The dynamics of revascularization can be monitored in Fetal liver kinase 1 (Flk1; also called VEGF-R2)-GFP::c-fms-like tyrosine kinase 1 (Flt1; also called VEGF-R1)-tdsRed double transgenic mice [15]. GFP and tdsRed fluorescence is observed within blood vessels, because Flk1 and Flt1 are both expressed in endothelial cells. We found that revascularization begins early after the ischemic insult. We also found the emerging pericytes first ensheath blood vessels and then produce fibroblast-like cells not in close contact with blood vessels.

2. Materials and Methods

2.1. Animals

We used male and female Flk1-GFP BAC transgenic mice, Flk1-GFP::Flt1-tdsRed BAC transgenic mice, and ICR wild type mice (10- to 12-week old). As we previously reported [13, 15], we used BAC clones that carry the whole Flk1 or Flt1 genomic region as well as the upstream and downstream regions. The GFP and tdsRed cDNA were introduced into the first exon of the Flk1 and Flt1 gene using the RED/ET recombination technique respectively. The resulting GFP and tdsRed carrying BAC transgene were injected into pronuclei to create Flk1-GFP and Flt1-tdsRed BAC Tg animals respectively. The expression level of baseline was high for both Flk1-GFP and Flt1-tdsRed, as shown in Fig. 2A. All procedures were performed in accordance with the guidelines for Animal Experimentation at Gunma University Graduate School of Medicine and were approved by Gunma University Ethics Committee (Permit Number:16-009).

2.2. Stereotactic ET-1/L-NAME injection

We injected endothelin-1 (ET-1) / N(G)-nitro-L-arginine methyl ester (L-NAME) into the internal capsule as previously described with some modifications [19]. Mice were anesthetized with isoflurane. Mice were placed in a stereotactic frame attached with 33G needle. For injection into the internal capsule, we set the needle tip on the bregma angled with 22°, moved the needle 0.8-1.1 mm posterior and 3.1-3.3 mm lateral left, and inserted the needle into the brain parenchyma 3.9-4.1 mm ventral from the skull surface angled with 22° keeping. Two microliters of ET-1 (100 pmol/µl, 1 µl, Peptide Institute, Japan) / L-NAME solution (2.7mg/ml, 1 µl, Sigma) mixture dissolved in phosphate buffered saline (PBS) were injected. The day of injection of ET-1/L-NAME was designated as 0 day post lesion (0 dpl).

2.3. Magnetic resonance imaging

1 Magnetic resonance (MR) imaging was carried out 1 day after the ET-1/L-NAME injection
2 by a 1-T benchtop MR scanner (Icon; Bruker Biospin, Germany). Mice were anesthetized with
3 isoflurane. T2-weighted image was used to detect the infarction area: repetition time 2000 ms, echo
4 time 85 ms, thickness 760 μm and interslice distance 760 μm.
5
6 *2.4. Cryosection*
7 Mice were anesthetized deeply with isoflurane and perfused transcardinally with 4%
8 paraformaldehyde in PBS. The brains were dissected and post-fixed with 4% paraformaldehyde in
9 PBS for 1 h, and were cryoprotected with 30% sucrose in PBS at 4°C. Frozen sections (20 μm in
10 thickness) were cut coronally using a cryostat (CM3050S; Leica Biosystems, Germany).
11
12 *2.5. Immunofluorescent staining*
13 The brain sections were fixed with 10% formalin neutral buffer solution, washed with PBS
14 containing 0.3% Triton X-100 (PBS-T), incubated with 2-3% bovine serum albumin (BSA) in PBS-
15 T for 60 min, and incubated with primary antibodies at 4°C overnight. Primary antibodies included
16 anti-GFP rabbit polyclonal (1:1000; MBL, Japan), anti-PDGFRβ goat polyclonal (1:100; R&D
17 systems, USA). After washing with PBS-T, sections were incubated with fluorophore-conjugated
18 secondary antibodies for 2 h at room temperature. Hoechst 33342 (1 μg/ml; Invitrogen) was used for
19 nuclear staining. For Ki-67 detection, sections were immunostained with an anti-GFP antibody
20 beforehand, fixed with 10% formalin neutral buffer solution again, heated in citrate buffer (pH 6) for
21 5 min using a microwave oven, incubated with 2% BSA in PBS-T, and incubated with an anti-Ki67
22 mouse monoclonal antibody (1:200; BD Pharmingen, USA). For fibronectin detection, sections were
23 immunostained with an anti-fibronectin rabbit polyclonal antibody (1:200; Abcam, United Kingdom)
24 using TSA system (Perkin Elmer, USA). Sections were observed using a fluorescence microscope
25 (BZ-X710, BZ-9000; Keyence, Japan) and confocal laser-scanning microscope (LSM880; Carl
26 Zeiss, Germany).

1

2 *2.6. Luxol fast blue staining*

3 To identify ischemic demyelinated lesions in the internal capsule, brain sections were

4 stained with Luxol fast blue (LFB) as previously described [17].

5

6 *2.7. Blood flow analysis*

7 Blood flow in and around the ET-1/L-NAME injected lesion was assessed by injection of

8 streptavidin-Alexa Fluor 350 (100 μg/100 μl) (Thermo Fisher Scientific, USA) - labeled biotinylated

9 *lycopersicon esculentum* (tomato) lectin (100 μg/100 μl) (Vector Laboratories, USA) transcardially.

10 Mice were anesthetized with isoflurane before intracardial injection of the labeling solution

11 described above. Five min later mice were perfused transcardially with 4% paraformaldehyde in

12 PBS, and cryosections were prepared as mentioned above.

13

14 *2.8. Quantitative real-time RT-PCR*

15 Fresh coronal sections (thickness; 1 mm) were cut with a brain slicer (Muromachi Kikai,

16 Japan). The internal capsule from two brain slices per one brain was dissected on an ice-cold metal

17 plate immediately. Total RNA was extracted using ISOGEN (Nippon gene, Japan). Quantitative PCR

18 was performed with Applied Biosystems 7500 Real-Time PCR System using SYBR Green PCR

19 master mix (Applied Biosystems) to validate the differences. The ΔΔCt method was used for relative

20 quantification of *Pdgfb* gene expression. *Gapdh* was used as the reference gene. Significant

21 differences were determined with a paired *t*-test. The primers used were: PDGF-B (sense, 5'-

22 CATCCGCTCCTTTGATGATCTT-3'; antisense, 5' -GTGCTCGGGTCATGTTCAAGT- 3'), GAPDH

23 (sense, 5'-ATGGTGAAGGTCGGTGTGAACG - 3'; antisense, 5'-

24 CGCTCCTGGAAGATGGTGATGG -3').

25

26 *2.9. Analysis of vessel area*

The area of Flt1-tdsRed-positive vessels and Flk1-GFP-strongly-positive vessels were measured using ImageJ software. Using LFB stained image, the ROI (region of interest) was defined on the demyelinated region in the internal capsule for the ipsilateral side, and on the internal capsule for contralateral side, respectively. The threshold of fluorescent intensity was determined to obtain binary images. The area covered by the blood vessel-like structures with Flt1-tdsRed fluorescence or Flk1-GFP strong fluorescence in the determined ROI was measured. Data are presented as the mean ± SD. The data were analyzed with freely available statistical software EZR.

3. Results and Discussion

3.1. ET-1/L-NAME injection induced demyelination in the internal capsule of mice

In previous studies, we induced white matter infarction in rat brains by ET-1 injection into the internal capsule, and found that transplantation of microvascular endothelial cells significantly promoted the recovery of the infarction [12, 17]. Since we needed to use Flk1-GFP::Flt1-tdsRed double transgenic mice to examine the changes in blood vessels in this study, we tried to induce white matter infarction by injecting ET-1 into mouse brains. ET-1 injection alone (80-100 pmol), however, did not stably induce white matter infarction in mice (Fig. S1). Hence we injected L-NAME as well as ET-1 into the internal capsule of mice as previously reported [19]. Injection of ET-1 and L-NAME together induced infarction in the mouse internal capsule. To follow infarction after the injection, we captured MR T2-weighed images 1 day after ET-1/L-NAME injection (1 day post lesion;1 dpl). MR imaging showed a high intensity area in the internal capsule (Fig. 1), indicating that focal infarction was induced in the internal capsule at 1 dpl. We performed LFB staining of brain sections made from the same mouse at 7 dpl (Fig. 1). The LFB staining at 7 dpl showed obvious demyelinated lesion at the same area as MR T2-weighted image showed. These data indicated that injection of ET-1/L-NAME together effectively induced focal infarction and demyelination in the internal capsule of mice.

3.2. Visualization of revascularization in white matter infarction

It was reported that the density of lectin-perfused vessels of the internal capsule was reduced by 85% 7 days after ET-1/L-NAME injection [19]. This indicates that small vessel constriction mediated by the ET-1/L-NAME injection was not recovered within 7 days. Hence we analyzed the dynamics of blood vessel loss and revascularization in the internal capsule after the white matter infarction within 7 days using the double transgenic mice (Flk1-GFP::Flt1-tdsRed tg mice) [15]. Matsumoto and others showed that Flt1-tdsRed expression overlapped well with that of Flk1-GFP, and Flt1 was expressed more abundantly in endothelial cells of large blood vessels such as dorsal aorta at developmental stage [15]. Furthermore, they showed Flt1 expression was seen in most endothelial cells but was relatively weak in newly forming endothelial sprouts, while Flk1 expression was more prominent in newly forming endothelial sprouts and relatively weak in dorsal aorta at embryonic stage. Therefore, we used Flk1-GFP::Flt1-tdsRed double Tg mice to monitor the preexisting blood vessels (visualized by tdsRed) and the angiogenic vessel growth (visualized by GFP) after the white matter infarction. The fluorescence of GFP and tdsRed changed throughout our experimental time course in the infarction area (Fig. 2A and 2B). The demyelinated lesions in the internal capsule determined by LFB staining (Fig. 1) were used to identify the white matter infarction region, and the changes in the blood vessels were analyzed in this area (Fig. 2). We saw Flt1-tdsRed-positive blood vessels on the contralateral side (Fig. 2A). These Flt1-tdsRed-positive blood vessels apparently decreased on the ET-1/L-NAME-injected side when compared to the contralateral (intact) side (Fig. 2B). By contrast, the Flk1-GFP-strongly-positive vessels were prominent only on the ipsilateral side, and they increased as time elapsed (Fig. 2B). We then quantified these changes by computer-based densitometric analysis. As seen in Fig. 2B, we always observed an increase in the basal level of green fluorescence in the infarct region. So we quantified the area covered by the blood vessel-like structures with Flt1-tdsRed or Flk1-GFP fluorescence. Consistent with the images (Fig. 2A and 2B), the area covered by Flt1-tdsRed-positive blood vessels on the ipsilateral side was

1 significantly decreased when compared to that on contralateral side at 1 dpl or 5 dpl (Fig. 2C).
2 Although there was no significant difference at 7 dpl, the reduction of the area covered by Flt1-
3 tdsRed-positive vessels on the ipsilateral side seemed to continue for a week (Fig. 2C), suggesting
4 that the vascularization was not recovered within a week. In contrast to the changes in the area
5 covered by Flt1-tdsRed-positive vessels, the area covered by Flk1-GFP-strongly-positive blood
6 vessels on the ipsilateral side increased when compared to that on the contralateral side in a time-
7 dependent manner (Fig. 2A, 2B, and 2D). These results indicate that the ET-1/L-NAME-injection
8 resulted in vascular loss at 1 dpl and induced angiogenesis from 3 dpl, and the angiogenesis still
9 remained active at 7 dpl.
10 We observed Flk1-GFP-strongly-positive blood vessels both in the periphery (Fig. 2B; 3
11 dpl, arrows) and the core (Fig. 2B; 7 dpl, arrows) of the internal capsule on the ipsilateral side. To
12 further examine the revascularization in the infarct area, we obtained images by a confocal laser-
13 scanning microscope (Fig. 3 and S2). We observed that Flk1-GFP-strongly-positive structures were
14 tubular (Fig. 3B, arrow) and branching (Fig. 3C, arrow). When we examined whether the blood flew
15 in the Flk1-GFP-strongly-positive vessels by fluorescence-labelled lectin perfusion at 7 dpl, it was
16 evident that the blood flew in some of these vessels, as some Flk1-GFP-strongly-positive vessels
17 were labelled by the lectin (Fig. 3D and 3E). Furthermore, we found Ki67-positive endothelial cells
18 in Flk1-GFP-strongly-positive vessels, suggesting that angiogenesis was occurring in this area (Fig.
19 3F and 3G).
20 Flk1 is the major mediator of the angiogenic effect exerted by VEGF-A [5]. Flt1 is also a
21 high affinity receptor for VEGF-A, but dispensable for vascular morphogenesis *in vivo*, presumably
22 acting as a decoy receptor [10, 16]. In the infarct region, we observed appearance of the Flk1-
23 strongly-positive blood vessels (Fig. 2B) from 3 dpl, indicating the occurrence of active angiogenesis
24 at this time point. Adamczak and others monitored the change of Flk1 expression in blood vessels
25 after temporary middle cerebral artery occlusion (MCAO) using Flk1-luciferase reporter mice, and
26 revealed that Flk1 expression started 3 days after the insult with peak values at 7 day [1]. This report

1 suggested that VEGF-Flk1 signaling might be important for angiogenesis after temporary MCAO. In
2 our study, Flk1-GFP-strongly-positive blood vessels on the ipsilateral side increased when compared
3 to that on the contralateral side within 7 days (Fig. 2). Their report and ours suggest that VEGF-Flk1
4 signaling might be the common regulator of angiogenesis after ischemia induced both by temporal
5 large artery occlusion and permanent occlusion in deep white matter by ET-1/L-NAME-injection.
6
7 *3.3. The PDGFRβ-strongly-positive cells increased in the infarct area*
8 We showed new blood vessels are formed in the core of white matter infarction in the acute
9 phase (Figs. 2 and 3). It is well known that the interaction between pericytes and endothelial cells is
10 essential for vessel stability and maturation [3]. Hence we examined the association between
11 pericytes and the blood vessels in the white matter infarction after ET-1/L-NAME injection (Fig. 4).
12 In this part, we used the Flk1-GFP BAC Tg mice and wild type mice, instead of the double
13 transgenic mice (Flk1-GFP::Flt1-tdsRed Tg mice) to visualize the pericytes "red" after
14 immunofluorescence staining for PDGFRβ, a marker for pericytes. The expression of PDGFRβ in
15 pericytes was very weak under physiological conditions (not shown). The PDGFRβ-strongly-positive
16 (PDGFRβ+) cells were not apparent at 1 dpl, but became prominent at 3 dpl and increased their
17 number thereafter in the infarct area (Fig. 4A). This result is consistent with the previous report that
18 PDGFRβ expression by the pericytes is upregulated in the MCAO model, while its expression is low
19 in the intact mature brain [4]. At 3 dpl, most of these cells were in close contact with Flk1-GFP-
20 positive endothelial cells, indicating these cells are bona fide pericytes (arrows in Fig. 4A). At 7 dpl,
21 the number of PDGFRβ+ cells increased dramatically, and some of these PDGFRβ+ cells ensheathed
22 the GFP-positive blood vessels (the rectangle in Fig. 4A is shown at higher magnification in B, and
23 arrows in B show pericytes ensheathing blood vessels). We quantified the area covered by PDGFRβ+
24 cells (Fig. 4C). Consistent with the images (Fig. 4A), the area covered by PDGFRβ+ cells in the
25 demyelinated lesion at 7dpl significantly increased compared to that at 1 dpl. These results indicate
26 that pericytes appeared together with newly formed blood vessels in the infarct area. Platelet-derived

1 growth factor B (PDGF-B), a ligand for PDGFRβ, is necessary for pericyte recruitment during
2 angiogenesis [3]. We analyzed the changes in the expression of PDGF-B in the white matter
3 infarction, and found that the expression of *pdgfb* mRNA increased on the ET-1/L-NAME-injected
4 side in the internal capsule when compared with the contralateral side (Fig. 4D). These data suggest a
5 possibility that PDGF-B/PDGFRβ signaling might regulate recruitment of pericytes to the infarct
6 area and contribute to the stabilization of new vessels in the white matter infarction. Further
7 experiments (e.g. examining effects of inhibition of this signaling on revascularization in the infarct)
8 are necessary to prove this possibility. Although we did not determine which cells produce PDGF-B
9 in the white matter infarction, two lines of evidence suggest that Flk1-GFP-strongly-positive cells
10 may produce it. First, PDGFRβ+ pericytes primarily appeared near the Flk1-GFP-strongly-positive
11 vessels on 3 dpl (Fig. 4). Second, it has been reported that PDGF-B is secreted by the migratory tip
12 cells at the leading edge of angiogenic sprout [8]. As shown in Fig. 4A, the vast majority of
13 PDGFRβ+ cells at 7 dpl were not in close contact with Flk1-GFP-positive endothelial cells. It has
14 been reported that PDGFRβ+ vascular mural cells proliferate extensively into fibroblast-like cells
15 that generate fibrous extracellular matrices (ECM) in MCAO model, spinal cord injury, and tumor [4,
16 6, 11]. The PDGFRβ+ cells also expressed fibronectin, a fibroblast marker, in the demyelinated
17 lesion at 7 dpl (Fig.4E). Hence there is a possibility that PDGFRβ+ cells not in close contact with
18 blood vessels might be involved in scar formation in white matter infarct.
19 In this study, we revealed revascularization begins early after the ischemic insult on the
20 white matter, and the emerging pericytes first ensheath blood vessels and then produce fibroblast-like
21 cells. The roles these fibroblast-like cells play in the recovery process of the white matter infarct
22 remain unclear at present and further study is required to answer this important question.
23

24 **Conflict of interest**

25 The authors declare that there are no actual or potential conflicts of interest related to the work

described in this report.

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Figure Legends

Fig. 1. The injection of ET-1/L-NAME into the internal capsule of mice induced white matter infarction.

ET-1/L-NAME was injected into the internal capsule of mice stereotactically. White matter infarction was confirmed by MR T2-weighed imaging (MR T2WI), and demyelinated lesion was determined by LFB staining in the same mouse. MR T2WI obtained 1 day after ET-1/L-NAME injection (1 day post lesion; 1 dpl) showed a high intensity area in the internal capsule. Arrow shows the high intensity area. The LFB staining at 7 dpl showed demyelination at the same area as MR T2WI showed. The area surrounded by the rectangle indicates the demyelinated lesion, and it is shown at higher magnification in the right panel. Scale bars: 1 mm, 200 μm (high magnification image).

Fig. 2. Dynamics of stable blood vessels and angiogenic blood vessels after white matter infarction. Focal white matter infarction was induced by stereotactic injection of ET-1/L-NAME into the internal capsule of Flk1-GFP::Flt1-tdsRed Tg mice. The brain sections were stained with an anti-GFP antibody at 1 dpl, 3 dpl, 5 dpl and 7 dpl. (A, B) The Flt1-tdsRed-positive blood vessels decreased in the ischemic lesion compared with contralateral (intact) side. We observed Flk1-GFP-strongly-positive blood vessels in the periphery at 3 dpl and in the core at 7 dpl of the internal capsule on the ipsilateral side. Arrows show the vessels visualized by Flk1-GFP. Dotted lines show the borders of internal capsule. Scale bars: 200 μm. (C, D) Quantification of the area covered by the blood vessels with Flt1-tdsRed (C) or Flk1-GFP fluorescence (D) after ET-1/L-NAME injection by using ImageJ. (C) The area covered by Flt1-tdsRed-positive-blood vessels on the ipsilateral side was significantly decreased when compared to that on the contralateral side at 1 dpl or 5 dpl (n= 3-5 mice on each day). $^{*}p < 0.05$, $^{\#}p < 0.01$ (paired *t*-test). The area on the ipsilateral side did not change significantly over time within 7 days. (one-way analysis of variance with Tukey-Kramer method) (D) The area covered by Flk1-GFP-strongly-positive vessels on the ipsilateral side was significantly increased when compared to that on contralateral side at 1 dpl, 5 dpl, and 7 dpl. $^{*}p < 0.05$, $^{\#}p<0.01$

1 (paired *t*-test). This area increased at 5 dpl and 7 dpl when compared with that at 1 dpl (n=3-5 mice
2 on each day). *$p < 0.05$, $^{\#}p<0.01$ (Games-Howell method).
3
4 **Fig. 3.** The structures of angiogenic vessels and the blood flow in them at the demyelinated lesion 7
5 days after ET-1/L-NAME injection.
6 (A-E) Focal white matter infarction was induced by stereotactic injection of ET-1/L-NAME into the
7 internal capsule of Flk1-GFP::Flt1-tdsRed Tg mice. Native fluorescence of GFP or tdsRed was
8 detected by confocal microscopy (B, C) or fluorescence microscopy (A, D, E) at 7 dpl. (A-C) Flk1-
9 GFP-positive vessels in the white matter infarction had tubular (arrow in B) and branching structures
10 (arrow in C). (D-E) Streptavidin-Alexa Fluor 350-labeled biotinylated *lycopersicon esculentum* lectin
11 was perfused transcardially at 7dpl. Arrow shows the Flk1-GFP-positive vessels labeled by the lectin.
12 (F, G) Focal white matter infarction was induced by stereotactic injection of ET-1/L-NAME into the
13 internal capsule of Flk1-GFP Tg mice. The brain sections were stained with an anti-GFP antibody
14 and an anti-Ki67 antibody and observed with confocal microscopy. Ki67-positive endothelial cells
15 were found in Flk1-GFP-positive vessels in the white matter infarction (arrowheads in F and G).
16 Scale Bars: 200 μm (A, D), 30 μm (B, C, E, F), and 10 μm (G).
17
18 **Fig. 4.** Dynamics of PDGFRβ-strongly-positive pericytes around the Flk1-GFP-strongly-positive
19 blood vessels in the white matter infarction.
20 Focal white matter infarction was induced by stereotactic injection of ET-1/L-NAME into the
21 internal capsule of Flk1-GFP Tg mice. (A, B) Tissue sections at 1 dpl, 3 dpl, and 7 dpl were
22 immunostained with an anti-GFP antibody, an anti-PDGFRβ antibody, and Hoechst 33342. The
23 PDGFRβ-strongly-positive (PDGFRβ+) cells were not apparent at 1 dpl, but became prominent at 3
24 dpl and increased their number thereafter in the infarct area (A). At 3 dpl, most of these cells were in
25 close contact with Flk1-GFP-strongly-positive endothelial cells, indicating these cells are bona fide
26 pericytes (arrows in A). At 7 dpl, the number of PDGFRβ+ cells increased dramatically, and some of

these PDGFRβ+ cells ensheathed the GFP-positive blood vessels (arrows in B). The vast majority of PDGFRβ+ cells at 7 dpl, however, were not in close contact with Flk1-GFP-strongly-positive endothelial cells. We obtained the similar results in 2-3 mice. (C) Quantification of the area covered by PDGFRβ+ cells after ET-1/L-NAME injection. The area covered by PDGFRβ+ cells in the demyelinated lesion at 7 dpl significantly increased compared to that at 1 dpl (n= 3 mice on each day). $*p < 0.05$ (Welch's *t*-test). (D) Quantitative PCR analysis showed the significant increase in *pdgfb* message in the internal capsule of the ET-1/L-NAME-injected side compared with that on the contralateral side. $^{\#}p<0.01$ (n= 3). (E) Focal white matter infarction was induced by stereotactic injection of ET-1/L-NAME into the internal capsule of wild type mice. Tissue sections at 7 dpl were immunostained with an anti-fibronectin antibody and an anti-PDGFRβ antibody. Nuclei were stained by Hoechst 33342. PDGFRβ+ cells also expressed fibronectin. Scale Bars: 200 µm (A), 100 µm (B), and 20 µm (E).

Supplementary information

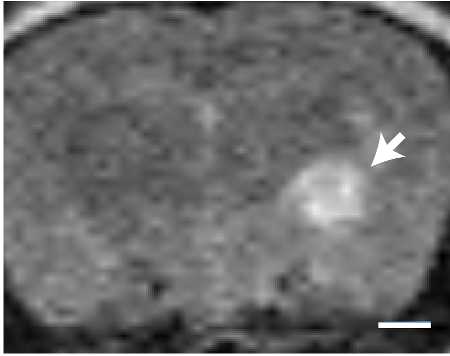
Figure S1. The injection of ET-1 into the internal capsule of mice did not stably induce white matter infarction. The LFB staining at 7 dpl showed only small demyelination area (arrowheads). Scale bar: 500 µm.

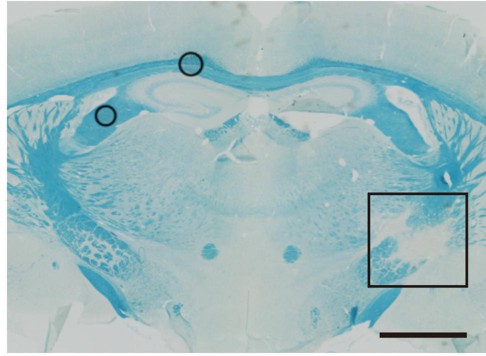
Figure S2. The structures of angiogenic vessels at the demyelinated lesion 7 days after ET-1/L-NAME injection. Native fluorescence of GFP or tdsRed was detected by confocal microscopy. Flk1-GFP-positive vessels in the white matter infarction had tubular (arrowheads in C, Z-stack image and Y-stack image) and branching structures (arrowhead in D, Z-stack image). Scale Bars: 100 µm (A, B), 10 µm (C) and 20 µm (D).

Highlights

•We induced the white matter infarction in the internal capsule of VEGFR2-GFP:: VEGFR1-tdsRed double transgenic mice.

1 •We followed expression of VEGFR2, a marker for angiogenic vessels, and VEGFR1, a marker for
2 preexisting vessels, in the white matter infarction.
3 •We found that revascularization begins early after the ischemic insult.
4 •We also found the emerging pericytes first ensheath blood vessels and then produce fibroblast-like
5 cells not in close contact with blood vessels.





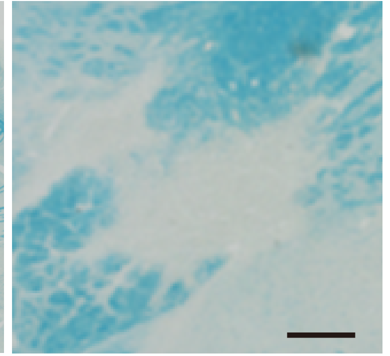


Figure 1 Shimauchi-Ohtaki et al.

Flt1-tdsRed / Flk1-GFP

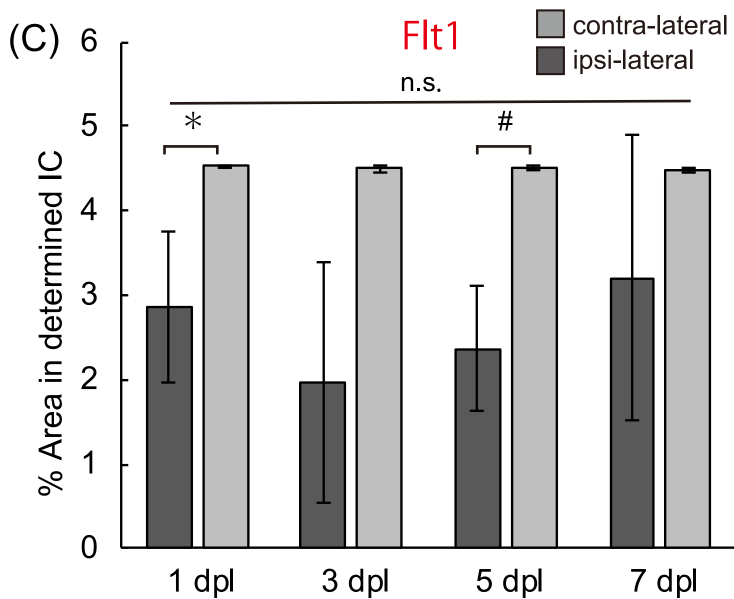
(A)▼ET-1/L-NAME (contra-lateral) (B)▼ET-1/L-NAME (ipsi-lateral)

1 dpl ▼

3 dpl ▼

5 dpl ▼

7 dpl ▼



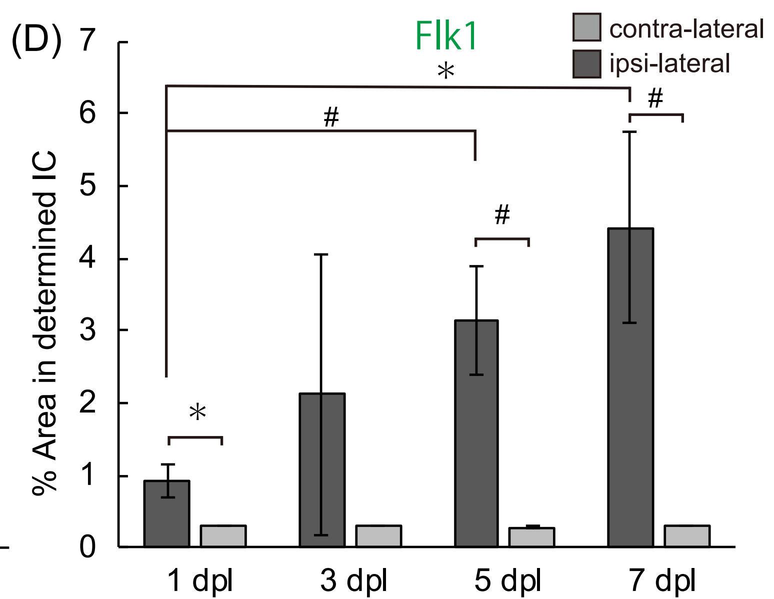


Figure 2 Shimauchi-Ohtaki et al.

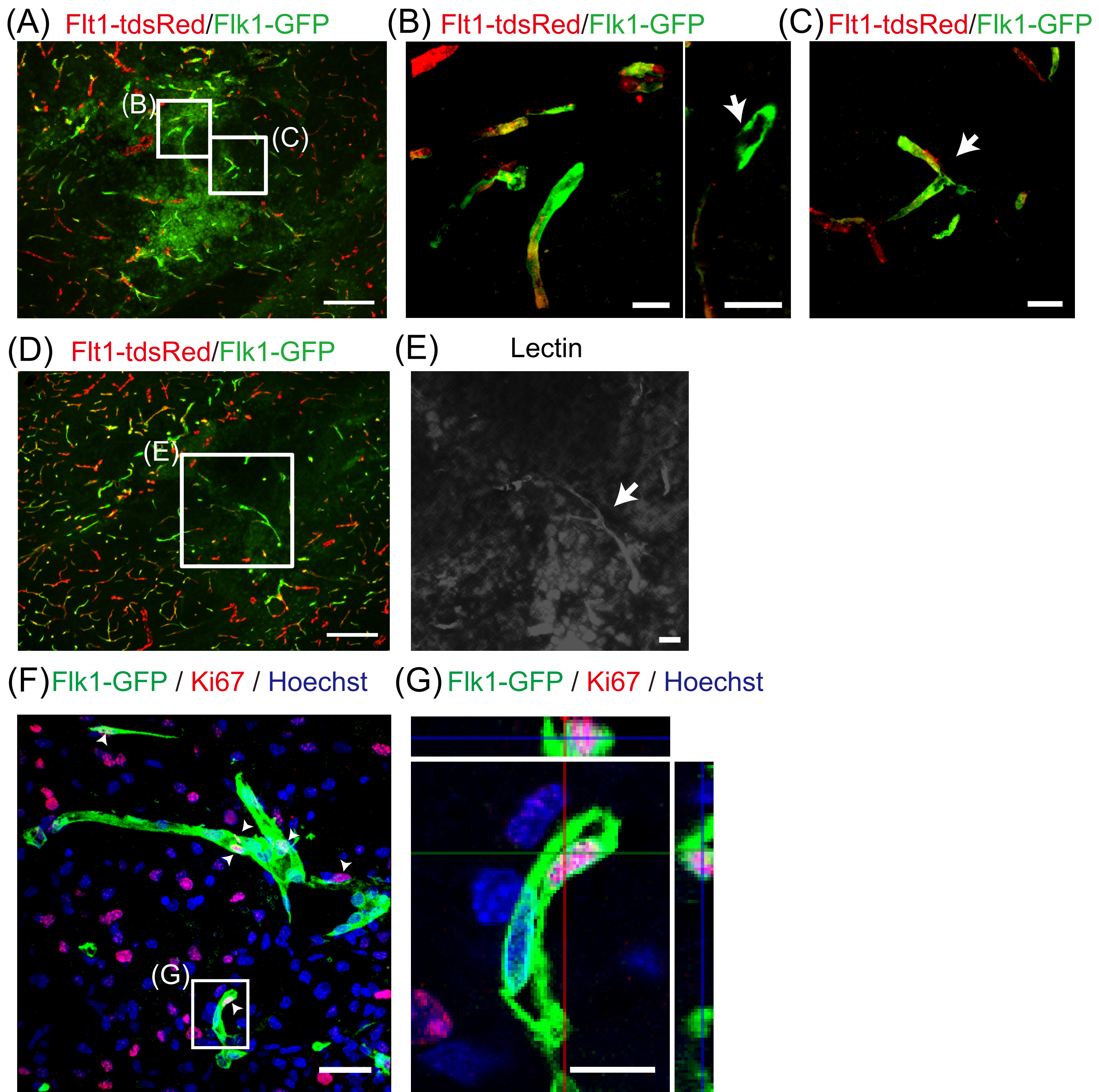


Figure 3 Shimauchi-Ohtaki et al.

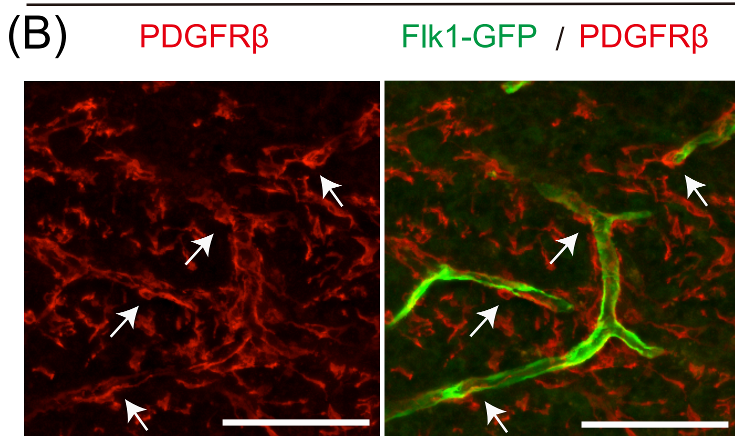
(A) ▼ Flk1-GFP ▼ PDGFRβ ▼ Flk1-GFP/PDGFRβ/Hoechst

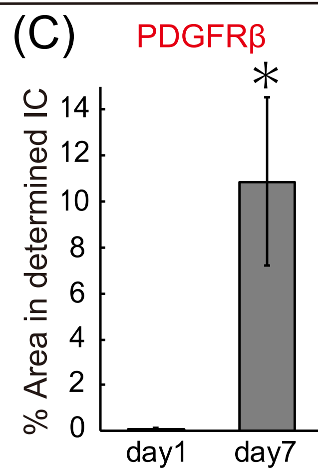
▼ 1 dpl

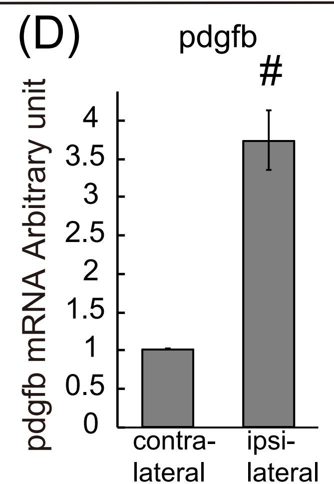
▼ 3 dpl

▼ 7 dpl

(B)







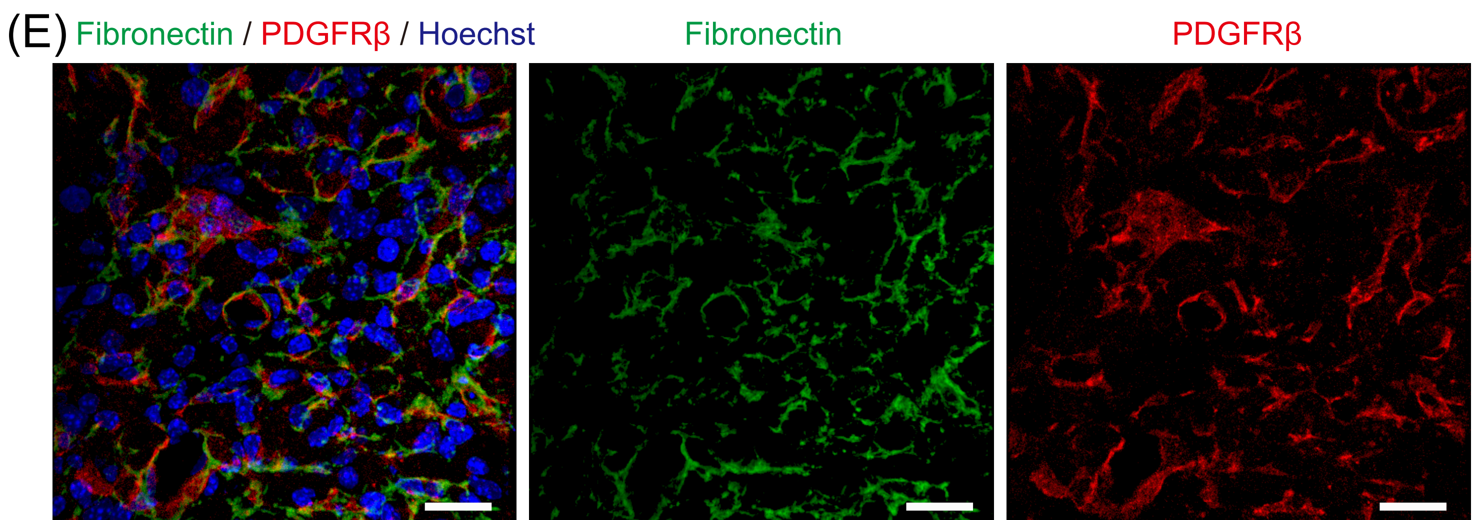


Figure 4 Shimauchi-Ohtaki et al.

LFB

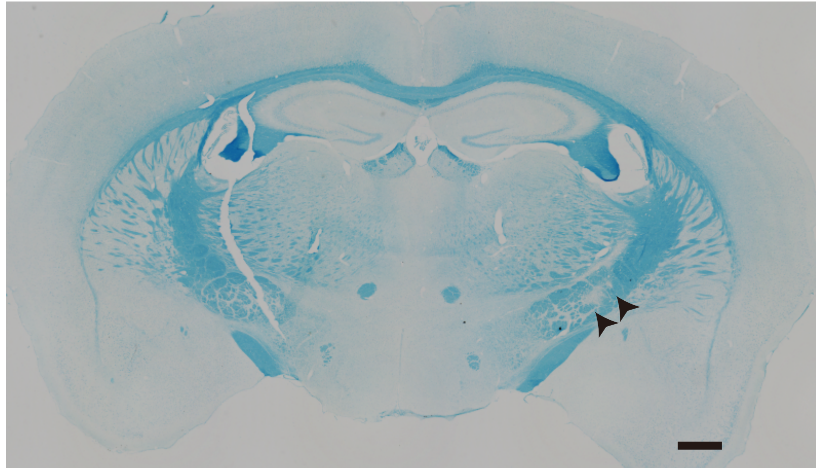


Figure S1 Shimauchi-Ohtaki et al.

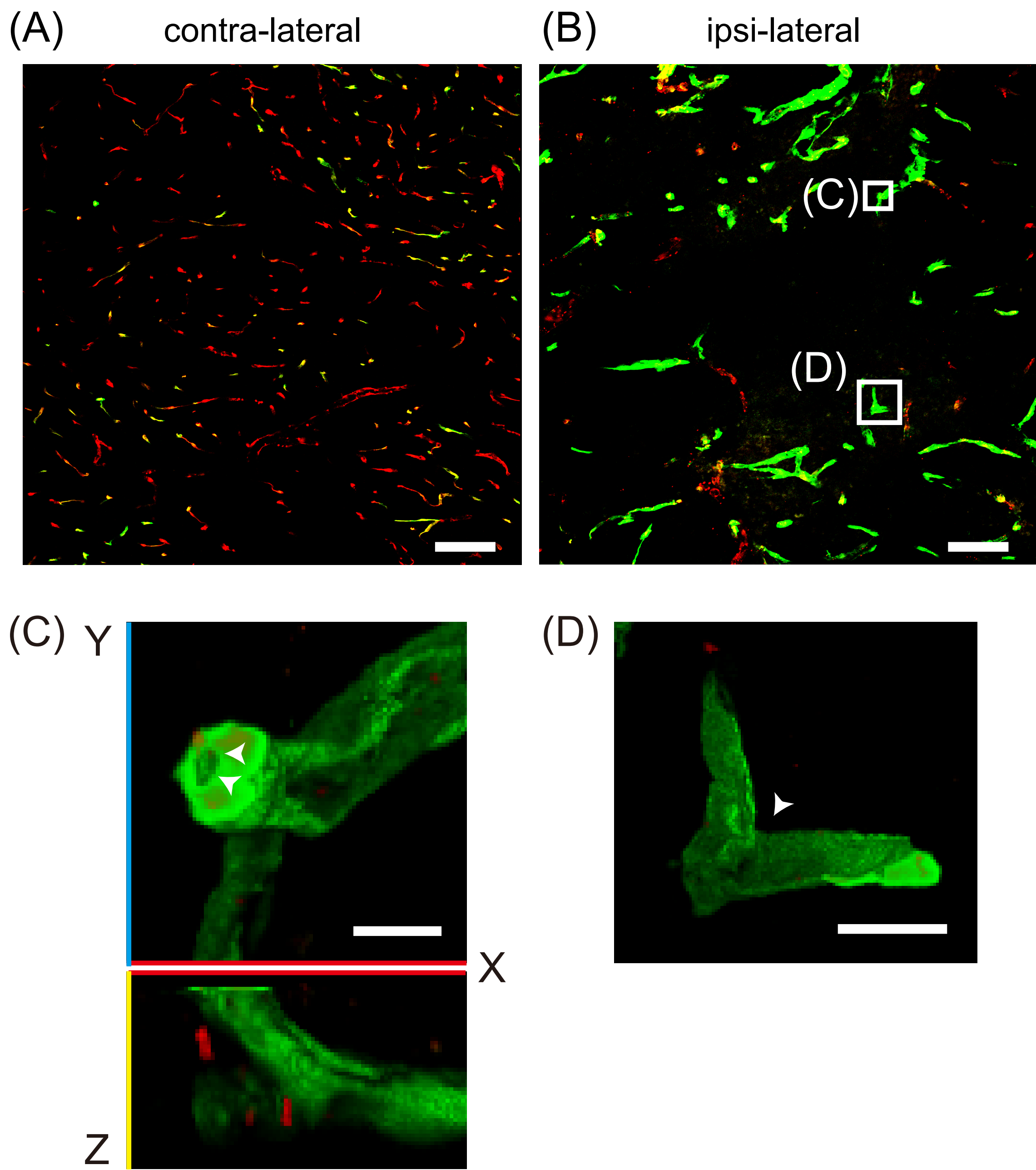


Figure S2 Shimauchi-Ohtaki et al.