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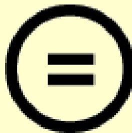

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藥學碩士學位論文

Ninjurin1 Contributes to Blood-Brain Barrier Protection in the Photothrombotic Stroke Model

Photothrombosis 뇌졸중 모델의 뇌혈관장벽 보호에 관여하는 Ninjurin1의 기능에 관한 연구

2015년 8월

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이 성 이

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이 논문을 약학석사학위논문으로 제출함

2015년 8월

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2015년 8월

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ABSTRACT

Ninjurin1 Contributes to Blood-Brain Barrier Protection in the Photothrombotic Stroke Model

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Nerve injury induced protein 1 (Ninjurin1, Ninj1) is a homophilic adhesion molecule that promote neurite extension via cell-cell interaction in the peripheral nervous system. In addition, Ninj1 mediates leukocyte trafficking in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. Previous studies were mainly investigated the function of Ninj1 in EAE pathogenesis. However, little is known about Ninj1's function in Central Nervous System(CNS).

Therefore, we now studied the role of Ninj1 on another CNS inflammatory disease such as ischemic stroke.

For making the ischemic stroke model, 8-10 weeks C57BL/6 mouse were subjected to ischemia by photothrombosis (PT). Using the custom-made Ninj1 antibody, we first examed the Ninj1 localization in normal and PT mouse brain. In normal brain, Ninj1 was expressed in macrophages of meninges. Interestingly, Ninjurin1 signals were dominantly detected in penumbra area surrounding the lesion core and weakly in endothelial cells in the lesion core after stroke in the early stage. The number of Ninjurin1 positive cells are increased in lesion core region according to stroke progression in the later stage.

To examine the Ninjurin1-expressing cell-types, we double-stained Ninjurin1 with immune cell markers such as CD45 (leukocyte), F4/80 (macrophage). Ninj1 was merged with CD45, F4/80 especially with F4/80. Furthermore, focal brain injury in Ninj1 KO mice led to the Blood-Brain Barrier(BBB) disruption as the lacking of Claudin5 and increased infarct volume in the recovery stage.

Altogether, we clarified that Ninjurin1 has beneficial roles for BBB protection by modulating the BBB intensity and distribution in macrophage of stroke tissue. Thus, our results suggest that Ninjurin1 could be a potent drug candidate for ischemic stroke treatments.

Keywords : Ninj1, Photothrombosis, Ischemic stroke, macrophage, Blood-Brain Barrier, CNS injury

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Ⅰ. INTRODUCTION

1. Ninjurin1

Nerve injury induced protein 1 (Ninjurin1, Ninj1) is a small homophilic adhesion protein composed of 152 amino acids. (Fig 1) It is located on the cell surface, and its expression is variable in many tissues such as epithelial cells, peripheral neurons and blood cells. Ninj1 promotes neurite extension of dorsal root ganglion neurons *in vitro*. (Araki et al., 1996, 1997). Later research revealed that Ninj1 expressed in perivascular macrophages of retina in developing mouse (Lee et al., 2009). Recently, It is identified that Ninj1 mediates leukocyte trafficking in the experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. (Ahn et al., 2014). It demonstrated that Ninj1 is also have its role in immune system in the central nervous system (CNS). However, little is known about the role of Ninj1 in the CNS.

Previous studies were mainly investigated the function of Ninj1 in EAE pathogenesis. Based on these results, we now studied the role of Ninj1 on another CNS inflammatory disease such as ischemic stroke.

2. Ischemic stroke and Photothrombosis model

Stroke is the second most common cause of death worldwide and

the leading cause of adult disability and caused by poor blood flow to the brain results in cell death (Murray and Lopez et al., 1997). Ischemic stroke occurs mainly because of occlusion of a cerebral artery by a thrombus. (Fig 2) It has enormous clinical, social, and economic implications and demands a significant effort from both basic scientists and clinicians in the quest for understanding the underlying pathogenetic mechanisms (Heuschmann et al., 2003). The ultimate result of ischemic cascade initiated by acute stroke is neuronal death along with an irreversible loss of neuronal function.

Furthermore, on the cellular level, the ischemia-induced breakdown of the blood-brain barrier (BBB), which is known to contribute to an increased risk of ischemic transformation (Sandoval KE et al., 2008).

Recently, the majority of ischemic stroke experiments are carried out in small animals such as the rat and the mouse. The mouse is the most commonly used animal in stroke studies because of many reasons including its resemblance to humans in cerebrovascular anatomy and physiology (Macrae et al., 1992).

Photothrombosis (PT) induces a cortical infarct by the systemic injection of a photoactive dye (Rose Bengal) in combination with irradiation by a light source at a specific wavelength (Watson et al., 1985). Generation of singlet oxygen leads to focal endothelial damage, platelet activation and aggregation in both pial and intraparenchymal vessels within the irradiated area (Dietrich et al., 1987b). (Fig 4) The region of irradiation can be determined so as to induce ischemic lesion in

any desired cortical area. Photothrombotic ischemic lesion lacks of penumbra because BBB breakdown in the lesion occur within minutes (Dietrich et al., 1987a).

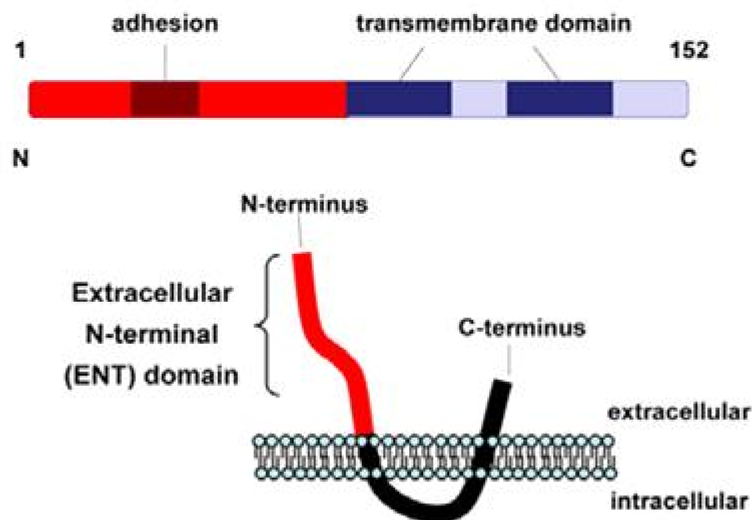
Experimental ischemic stroke models contribute to our understanding of the events occurring in ischemic brain.

3. Blood-Brain Barrier

The blood - brain barrier (BBB) is primarily formed by specialized brain endothelial cells that are interconnected by tight junctions and provides a dynamic interface between the blood and the brain (Abbott et al., 2010). (Fig 3) BBB disruption is a critical event in the pathogenesis of acute ischemic stroke. However, the molecular mechanisms involved are not completely understood.

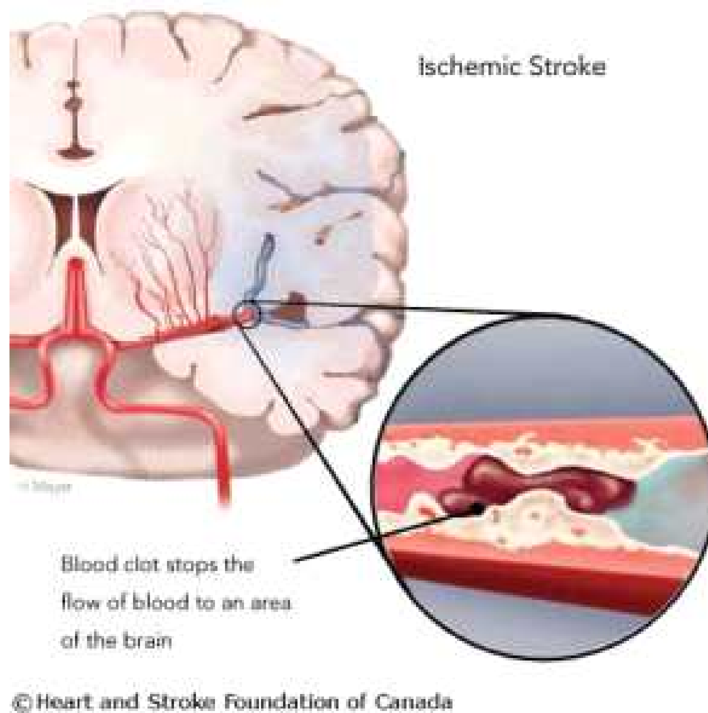
Under ischemic stroke conditions decreased BBB Tight Junction (TJ) integrity results in increased paracellular permeability, directly contributing to cerebral vasogenic edema, hemorrhagic transformation, and increased mortality (Sandoval and Witt et al., 2008).

Following ischemic stroke, the integrity of the BBB, which prevents harmful substances such as inflammatory molecules from entering the brain, can be impaired in cerebral areas distant from initial ischemic insult.



- **Ninj1 is composed of 152 amino acids**
- **Two transmembrane domains**
- **Homotypic adhesion molecule**

Figure 1. The Structure of Ninjurin1. Ninjurin1 is an adhesion molecule composed of 152 amino acids. It contains N-terminal (1-71 aa), C-terminal (139-152 aa), two transmembrane domains (72-100 aa and 111-138 aa) and intracellular domain (101-110 aa). N-terminal domain of Ninjurin1 mediates homophilic binding.



Heart and Stroke Foundation of Canada

Figure 2. Pathogenesis of Ischemic stroke. A Ischemic Stroke is a sudden loss of function due to loss of blood supply to an area of the brain that controls that function. It is usually caused by partial or complete blockage of an artery that supplies the brain. This clot may break loose and travel to an artery in the brain where it becomes lodged and totally blocks blood flow, causing permanent damage.

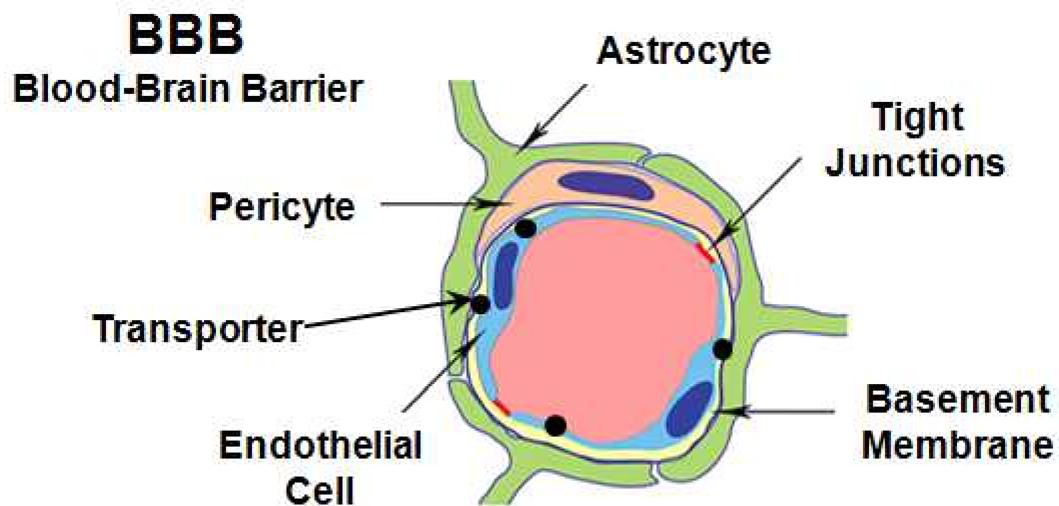


Figure 3. A schematic of Blood-Brain Barrier. The blood - brain barrier is formed by brain microvascular endothelial cells, astrocytes and pericytes. It maintains the neural microenvironment by regulating the passage of molecules into and out of the brain, and protects the brain from any microorganisms and toxins that are circulating in the blood.

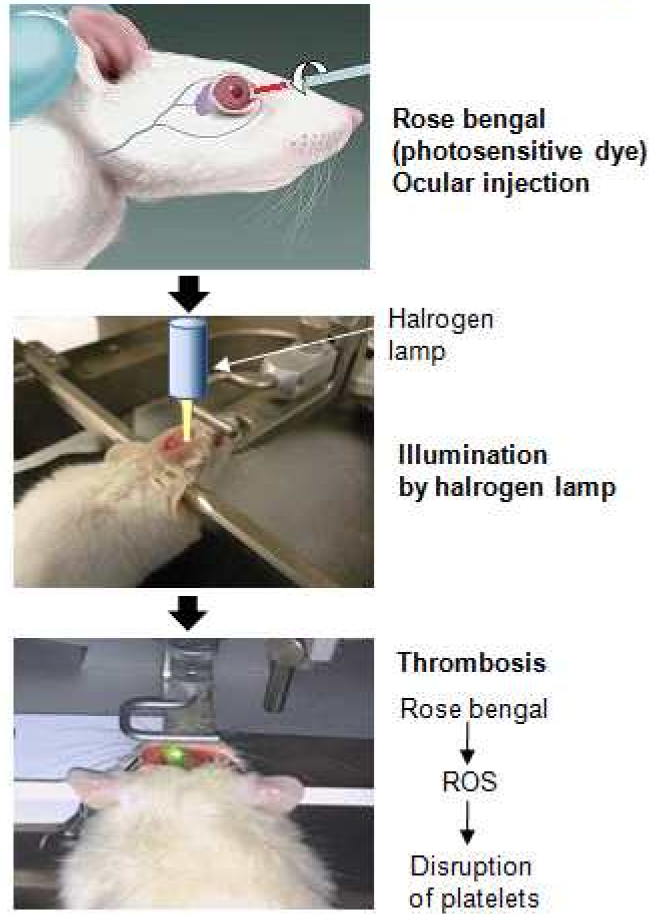


Figure 4. Photothrombosis model as a brain focal injury model

The WT and Ninj1 KO mouse (6weeks, 20-15g) were subjected to photothrombosis. Under intraperitoneal anesthesia, Rose Bengal was injected via ocular vein, which was allowed to circulate for 5 min. After the incision of scalp, the skull was illuminated by halrogen ramp for 20 min. When illuminated by halrogen ramp, the dye becomes activated and induces endothelial damage with platelet activation and thrombosis, resulting in local blood flow disruption.

Ⅱ. MATERIALS AND METHODS

1. Animals

C57BL/6 were purchased from Orient Bio. Inc., Korea. Ninjurin1 KO mice (C57BL/6J background) were generated by Dr. Goo taeg Oh at Ewha Womans University, Korea and backcrossed with C57BL/6 for at least seven generations. The breeding colonies were established and maintained under pathogen-free conditions in the animal housing facility of the College of Pharmacy, Seoul National University, for the duration of the experiments under the rule of the Committee for Care and Use of Laboratory Animals at Seoul National University (SNU-140630-5).

2. Photothrombosis Induction

WT and Ninj1 KO mice (C57BL/6 background, weighing 20-25 g) were subjected to PT. Under anesthesia by intraperitoneal injection of Zoletil (30 mg/kg) and Rompun (10 mg/kg), Rose Bengal (Sigma, 0.1 ml/25g body weight of 10% solution) was injected via the ocular vein, which was allowed to circulate for 10 min. After an incision in the scalp was made, the skull was exposed to a cold light source (Zeiss FL1500

LCD, 150W, 1mm diameter) for 20 min. The position of the optic center was 2.5 mm to the left and 2.5mm to the back of the bregma. Body temperature was maintained at 36 - 38 °C using a heating pad. The scalp was satured and the mice were allowed to regain consciousness.

3. Antibodies

For a custom-made anti-mouse Ninj1 antibody, keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide-bearing mouse Ninj1 residues 1-15 (Ab 1-15) or 139-152 (Ab 139-152) were immunized to rabbits following standard procedures (Peptrom Inc. and Abfrontier Inc., Korea); these anti-Ninjurin1 antibodies were purified in each case with antigen-specific affinity chromatography. Ninj1 antibodies was used for immunostaining (Ab 1-15) and Western blotting (Ab 1-15) and 139-152 (Ab 139-152).

4. Western blotting

The lesion tissues from PT mice were homogenized and incubated for 30min at 4°C in cell lysis buffer [50mM Tris-HCL (pH 7.5), 150mM NaCl, 1mM EDTA, 1% nonyl phenoxypolyethoxylethanol-40, 0.25%

Deoxycholate, and protease inhibitor cocktail]. Immunoblotting was performed using primary antibodies specific for Ninj1 (Ab 1-15 and Ab 139-152), Actin (Sigma), F4/80 (Serotec).

5. Nucleic acid extraction and Reverse transcription

Normal brain tissue and PT induced brain were homogenized mechanically with operating scissor for 1 min in Trizol (Invitrogen). The RNA concentration and purity were assessed with the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific). Samples were then stored at -80°C. 1ug total RNA was reverse-transcribed to produce cDNA using random and oligo-dT primers and superscript reverse transcriptase (Invitrogen Life technologies) in total reaction volumes of 20ul. All cDNA samples were diluted 10 fold with distilled waster and stored at -20°C.

6. Polymerase Chain Reaction

All amplications were performed in a total reaction volume of 50ul containing.

PCR was performed in a T3000 thermocycler(Biometra) and amplifications were performed using an initial denaturation temperature of

94 °C for 5 min followed by 35 cycles of annealing and extension at 58°C and 72°C respectively. The final extension was at 72°C for 10 min. Amplified products were visualized by a computerized image analysis system. PCR products with molecular size 500 bp were considered for Ninjurin1 and GAPDH.

The sequences of the primers were

Ninjurin1 (1-79) R : 5'-CCAGCGGCCGCCTAATGGTGATGGTGATGATGGGGCACGAAGAAGGCGAAAT-3′

Ninjurin1 (1-79) F : 5'-CTCGAATTCATGGGAGTCAAAGTTCTGTTTGCCCTGATCTGCATCGCTGTGGCCGAGGCCATGGAGTCGGGCACTGAGGA-3'

GAPDH R : 5'-TCCACCACCCTGTTGCTGTA-3'

GAPDH F : 5'-ACCACAGTCCATGCCATCAC-3'.

7. Immunofluorescence

Anesthetized mice were perfused with 0.1M PBS (pH 7.4) and the PT brains were isolated. Isolated brains were fixed with 4% paraformaldegyde at 4°C and dehydrate with 30% sucrose in PBS. Brains were embedded in OCT compound blocks and frozened under -80°C condition. Cryostat sections of 10um attached to microslides. Brain sections were rinsed in PBS to remove OCT compound for 30 min. Samples were blocked with blocking buffer composed of 10% fetal bovine

serum(FBS) and 0.05% bovine serum albumin(BSA) and 0.3% trixon X-100 in PBS and stained with primary antibodies to Ninjurin1, F4/80, CD45, Laminin, CD31 and Claudin 5 at 4 °C overnight. After washing three times with PBS, incubation with Alexa 488-conjugated IgG or Alexa 546-conjugated IgG as secondary antibodies at room temperature for 1 hour. Samples were washed three times with PBS and were mounted with gel/mount M01 solution. Images were obtained using LSM700 confocal microscopy (Carl Zeiss).

8. Evans blue permeability assay

The mouse was anesthetized by intraperitoneal injection of Zoletil (30 mg/kg) and Rompun (10 mg/kg). Evans blue solution (Sigma, 4% in PBS; 4mL/kg) was injected via the ocular vein. After circulation for 2 hr, the mice were intra-cardially perfused with 50 ml of ice-cold PBS, the brain tissue was extracted and divided into right and left hemispheres. Each hemisphere was homogenized (Tissue tearor, 50s; speed 35) in 1 mL (total volume) of 0.1 M PBS. The supernatant was collected and an equal amount of tricholoracetic acid solution (Sigma, 50% in PBS) was added. After incubated over night at 4 °C, the solution was centrifuged (30 min, 15,000 rpm, 4°C). Supernatant was extracted and measured by spectrophotometer at 610 nm.

9. Triphenyltetrazolium chloride (TTC) staining

The mice were intra-cardially perfused with 50 ml of ice-cold PBS, the brain tissue was extracted. Brains were cut into 2mm thick coronal sections with brain slicer. Brain slices near lesion site were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma) for 30 min at 37 °C. After washing in PBS, stained slices were fixed in 4% paraformaldehyde solution for 30 min. The size of the infarct area, which was divoid of red staining, was analyzed with image analysis software Image J (NIH, USA)

10. Data analysis and statistics

Quantification of band intensity was analyzed using Image J (NIH, USA). Statistical analysis was calculated using an unpaired two-tailed Student t-test for single comparisions and an ANOVA test for multiple comparisions $*P < 0.05$ was considered statistically significant.

Ⅲ. RESULTS

1. Ninjurin1 was highly expressed in infarct lesion of photothrombosis brain

To investigate the Nin1 expression level in infarct lesion during the ischemic stroke *in vivo*, the brain tissues of PT induced mouse, the animal of ischemic stroke, were examined. When RT-PCR was performed using Ninj1 primers, the mRNA level of Ninj1 in PT brain tissues was significantly increased compared to Wild-Type (WT) tissue.

To confirm more exactly we have conducted western blot analysis. The western blot data also shown the similar tendency in protein level. In Ninjurin1 Knock-Out (KO) tissue, Ninj1 expression was markedly increased expressed compared to WT tissue. Base on these results, we concluded that Ninj1 was expressed in infarct lesion of PT brain and was up-regulated in ischemic stroke condition, we presumed that Ninj1 is increased in the part of brain injury region.

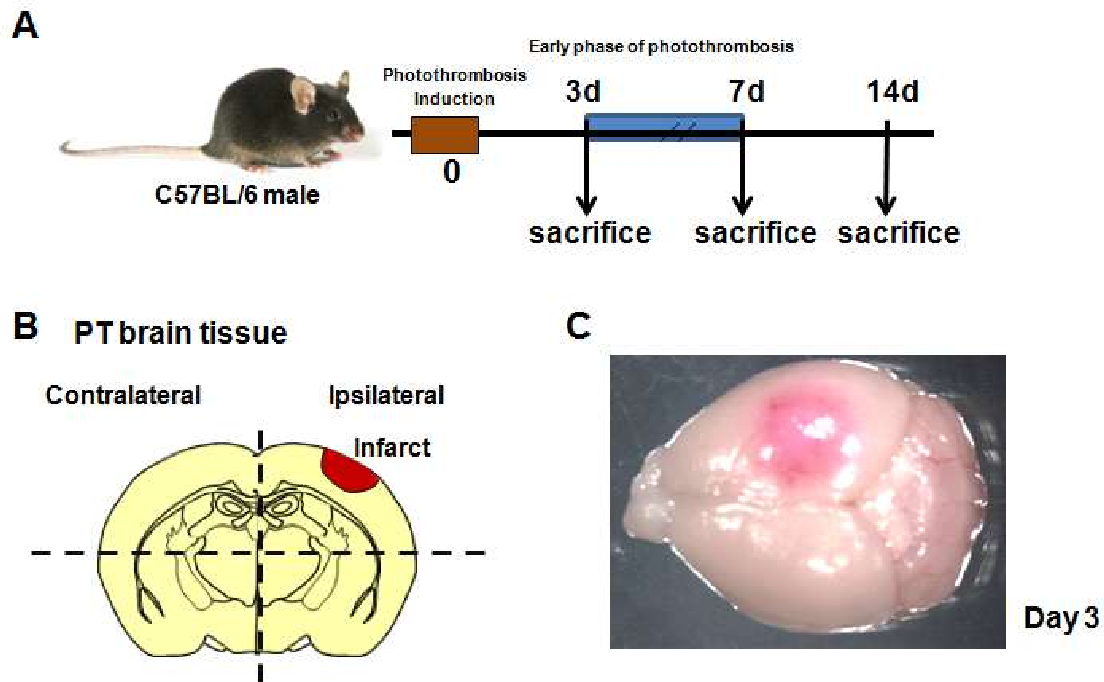


Figure 5. Induction of photothromobotic injury and the histological characteristics of the infarct area. (A) Schematic diagram of the experimental procedure. C57BL/6 mice, weighing 20-25g, were subjected to PT. After ischemic stroke induction, mouse brain was harvested at early phase of PT mice was sacrificed from day 3 to day 7. (B) Schematic representation of PT brain tissue. (C) After photothrombotic injury induction, the lesion site was easily distinguishable from the normal tissue. Injured mouse brain tissue was red compared to adjacent tissues.

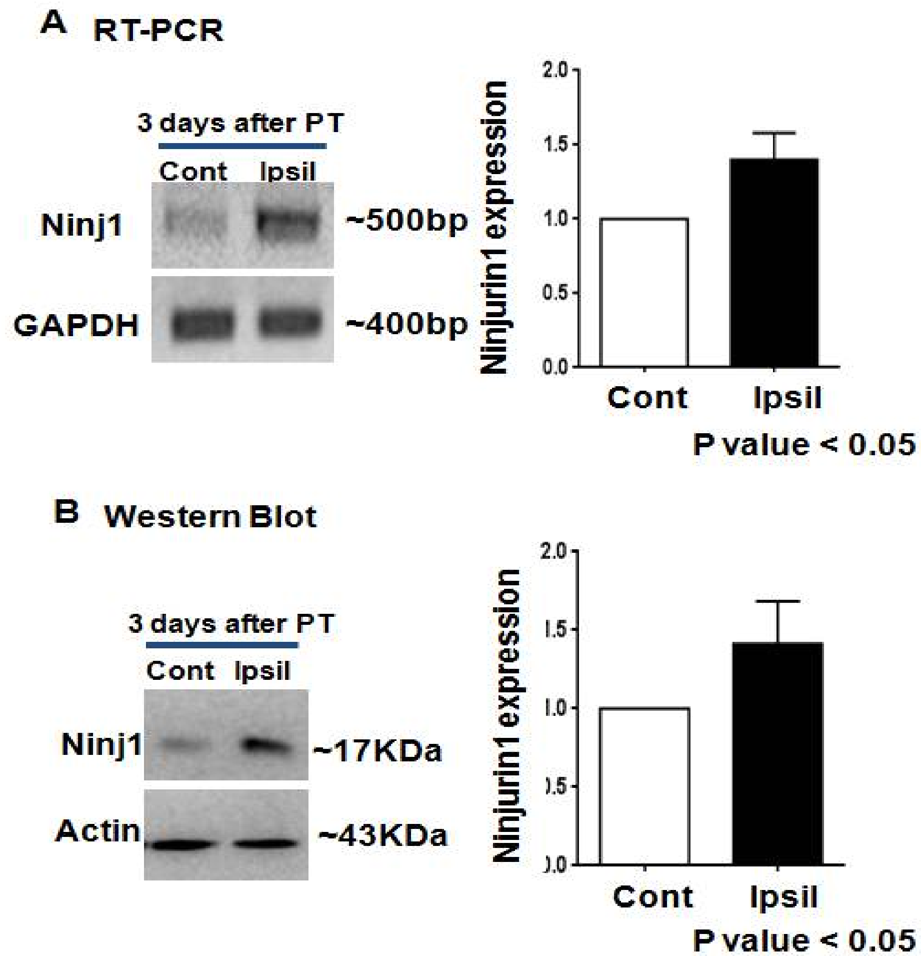
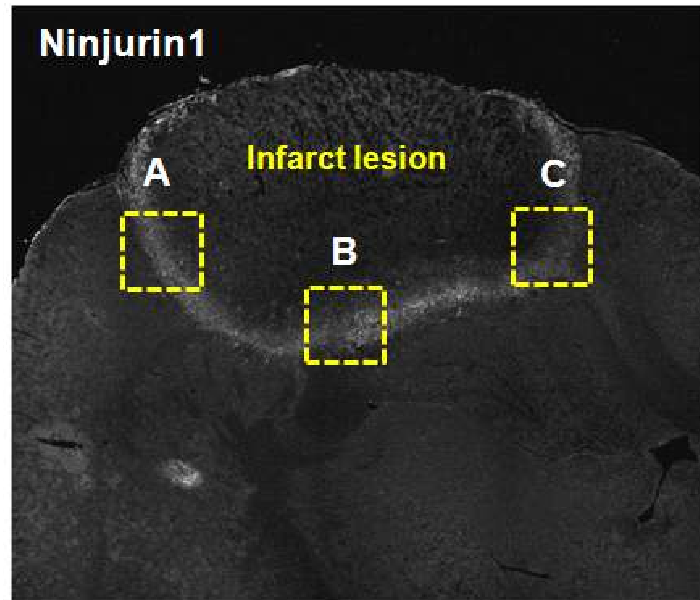


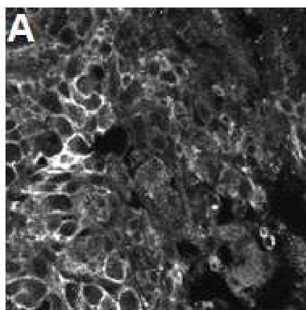
Figure 6. Ninjurin1 was highly expressed in the photothrombotic injury. The lesion tissue and normal tissue were harvested from the brain of mice at 3 days after PT induction and the tissue was analyzed by RT-PCR and Western blot. (A) RT-PCR analysis of Ninj1 expression in the photothrombotic area compared to controlateral cortex area. GAPDH was used as a loading control. Relative levels of Ninj1 mRNA (Right graph). (B) Western blot analysis of Ninj1 expression also showed the similar tendency in protein level . Actin was used as a loading control. Relative levels of Ninj1 protein (Right graph). (*$P<0.05$, t-test, n=3).

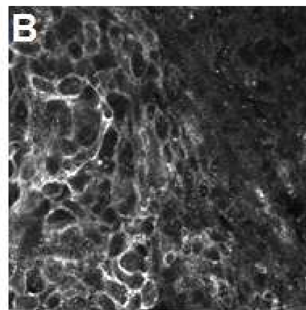
2. Ninjurin1 signal was dominantly detected in penumbra area surrounding the lesion core after photothrombosis in the early phase

To know what is the expression type of Ninj1 after photothrombotic injury, we performed immunohistochemistry on brain sections with ischemic damage induced by the halogen light source, with an irradiated area of 2.5 mm in diameter. Brain sections were immunostained with an antibody against Ninj1. As shown in Figure 7, round-shaped Ninj1 signal was clearly seen in the penumbra area of infarct region at 5 days after PT induction. We also observed the increased expression level of Ninj1 in the penumbra area, indicating the activation of Ninj1 after PT induction. These phenomena were observed in all mice subjected to immunohistochemistry examination.

Taken together, the expression of Ninj1 is up-regulated during ischemic stroke in vivo and dominantly detected in penumbra area surrounding the lesion core in the early phase of PT.







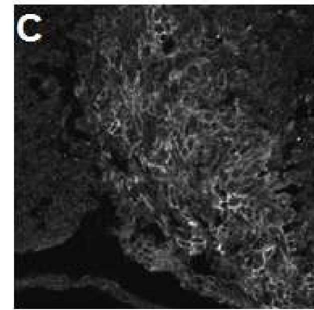


Figure 7. Upregulation of Ninjurin1 in the penumbra area of phothothrombosis. Brain sections were immunostained with antibodies for Ninj1. The rounded-shape Ninj1 was detectable in the penumbra region. (Magnified images of upper panel)

3. Ninjurin1 was expressed in myeloid cells in the penumbra of phoththrombosis brain

To determine the major cell types of Ninj1 expressing cells, various immune cell markers with Ninj1 double-staining of PT brain tissues was performed. CD45 was used as a pan leukocyte marker, and its expression merged highly with that of Ninj1. F4/80 is a myeloid cell marker, which can stain macrophage. From the results, Ninj1 was co-localized with myeloid cell especially with macrophage in WT tissue. However, Ninj1 signals in this cell types did not detected in the Ninj1 KO tissue. The macrophage near the penumbra area has an enlarged cell body and suggests the activation of macrophage after photothrombotic ischemic injury. The western blot analysis revealed similar results as well for the expression of macrophage increased in ipsilateral part compared to controlateral part.

Under photothrombotic ischemic injury condition, macrophage was significantly up-regulated, and much more macrophages in ipsilateral part as compared with the controlateral part in WT mice.

These results demonstrated that ischemic stroke induces a fast and sharp increase of macrophage in the penumbra region. Overall, the density of Ninj1 merged with macrophage cells in ipsilateral part is higher than the controlateral part in early phase after PT.

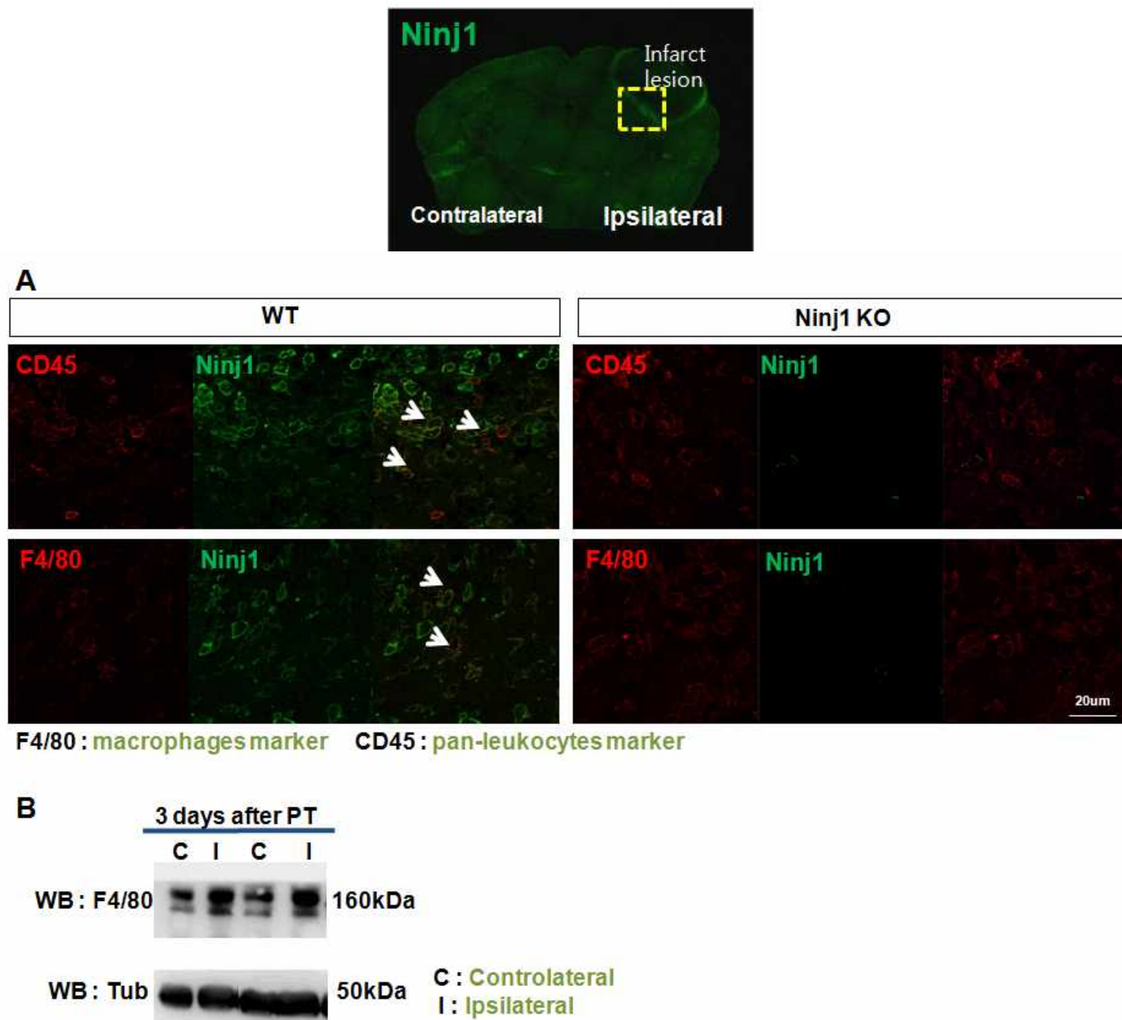
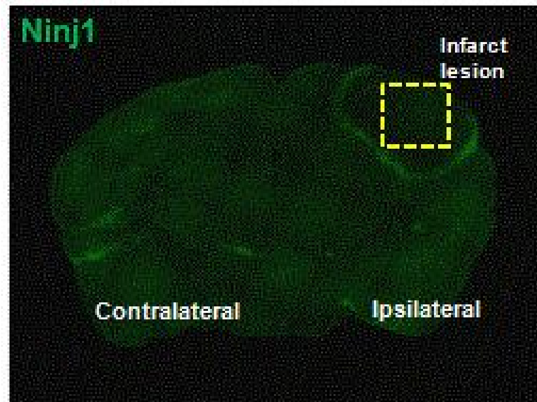


Figure 8. The identification of Ninjurin1-positive cell in the penumbra area and macrophage expression in photothrombotic injury. (A) Double-immunostaining of Ninj1 with CD45 (pan-leukocyte marker) and F4/80 (macrophage marker) in the penumbra area of brain tissue at 3 days. White arrow indicates Ninj1 positive myeloid cell especially in macrophage. (B) Cortex of controlateral and ipsilateral were subjected to Western blot analysis performed using F4/80 antibody.

4. Ninjurin1 was weakly expressed in endothelial cells in the lesion core of photothrombosis brain

To investigate the expression of Ninj1 in the lesion core of PT brain, we performed double immunostaining using primary anti-Ninjurin1 antibody with the specific markers for endothelial cells (CD31 and Laminin).

Ninj1 expression was weakly merged with endothelial cell in the lesion core of PT. Therefore, these results suggest the possibility that ninj1-mediated homophilic binding exists and plays a role in cell-cell interaction between leukocyte and brain endothelial cells in PT mouse, resulting in the entry of macrophage into the injury area across the BBB.



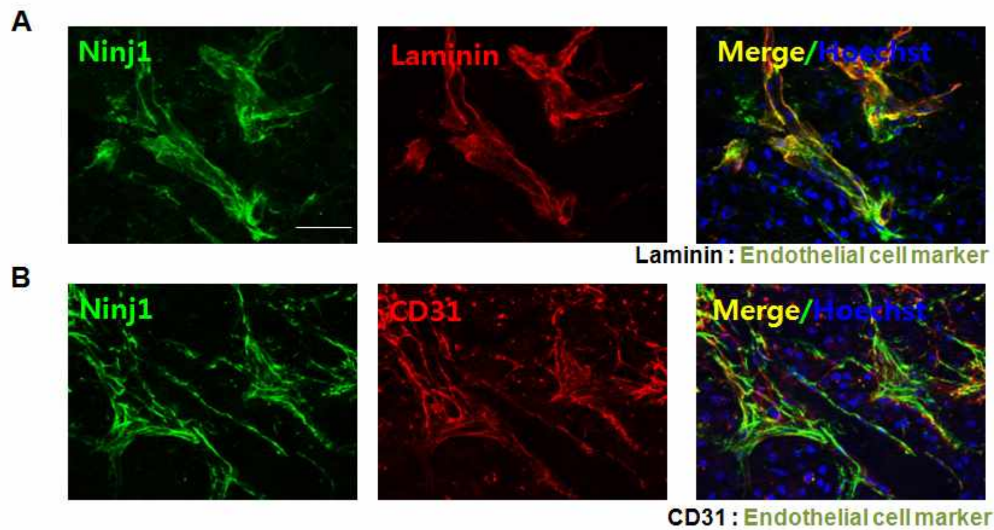


Figure 9. Ninjurin1 was weakly expressed in endothelial cells in the lesion core of photothrombosis brain. Higher magnification images (Yellow box) of the lesion core of photothrombotic injury tissue.

(A, B) The endothelial cell markers (Laminin, CD31) is co-localized with Ninj1 in the lesion core of photothrombotic brain tissue.

5. Blood-Brain Barrier leakage was more severe in Ninjurin1 KO mice than WT

The integrity of the BBB was investigated using Evans Blue extravasation. Evans blue was used as an indicator of increased BBB following PT in mouse models of stroke. Evans blue (4% in PBS, 4ml/kg) was injected intravenously after the onset of PT.

Evans blue dye leakage appeared to be concentrated mostly in the central regions of ischemic cortex in early phase. In contrast, Evans blue leakage after PT day 3 was observed throughout the ischemic cortex. As the figure 10 shown, the BBB leakage seemed to have mainly recovered after day 7. Therefore, the evans blue assay has been used for the quantification of BBB breakage, we can measure the BBB integrity after PT.

The distribution of Evans blue dye leakage in Ninj1 KO mouse that underwent photothrombotic injury was more severe than WT. Evan blue dye in WT mouse demonstrated that leakage within the peripheral ischemic areas of the cortex was significantly higher compared with Ninj1 KO mouse.

Furthermore, brain tissues of WT and Ninj1 KO after day 3 and day 14 of photothrombotic injury were stained with Claudin 5. Claudin 5 signal was completely higher from the WT brain than in the Ninj1 KO brain endothelial cells.

These results demonstrate that Ninj1 may have a role of BBB protection through the Claudin 5 tight junction protein that blocked the extravasation of vascular derived substances into the brain.

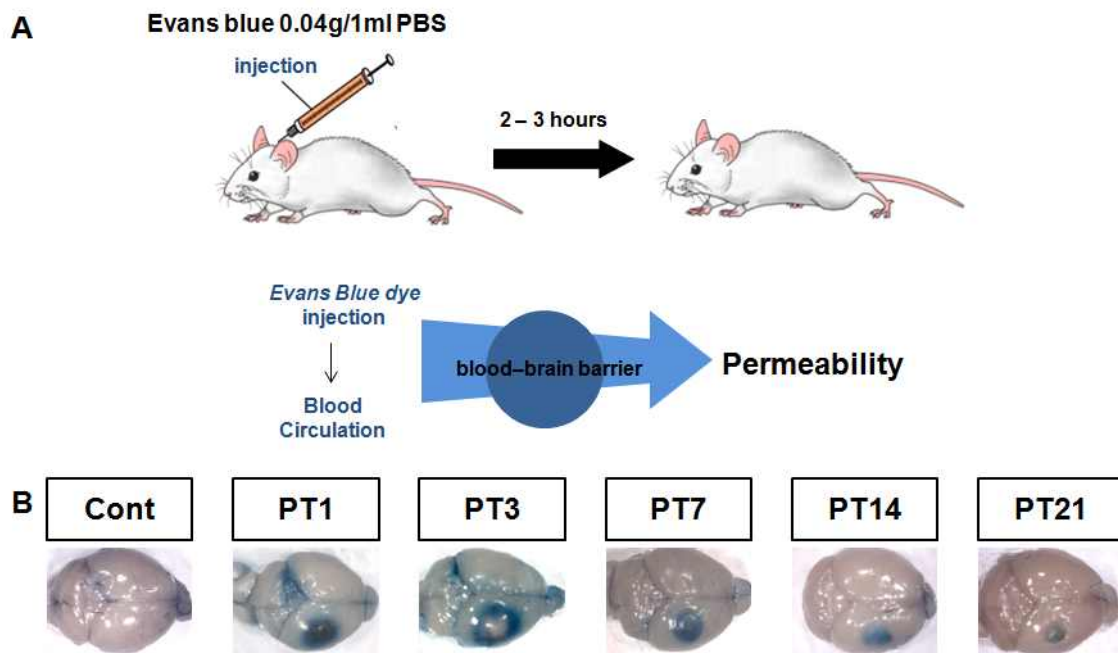


Figure 10. Evans Blue Assay for study blood-brain barrier disruption.

(A) Schematic diagram of experimental procedure.

(B) Evans blue extravasation assay at each time point.

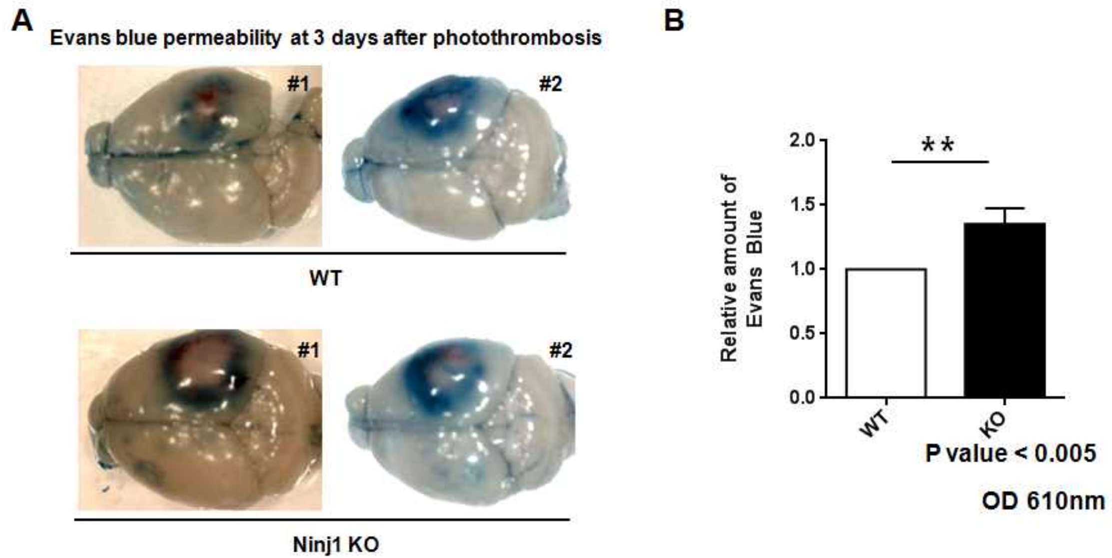


Figure 11. BBB leakage of Ninjurin KO mice was increased compared to WT mice.

Blood-Brain Barrier disruption in early stage of photothrombotic focal cerebral ischemia in the mouse was more severe in Ninj1 KO mice. (A) Analysis of Evans blue dye extravasation in WT and Ninj1 KO mice. Brains were isolated and photographed 3 days after PT induction. (B) Relative quantification of Evans blue permeability.

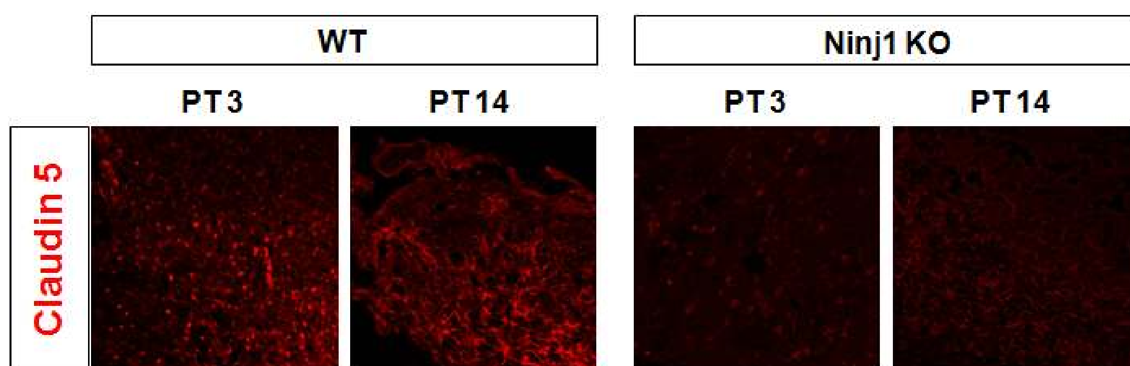


Figure 12. Claudin 5 in Brain blood vessels.

Brain tissues of WT and Ninj1 KO after day 3 and day 14 of photothrombotic injury were stained with Claudin 5. Claudin 5 was completely detectable from the wild type brain but not in the Ninj1 KO brain endothelial cells.

6. Ninjurin KO mice showed larger infarcted tissue compared to WT in the late phase of photothrombosis

TTC staining was used to measure the infarct volume after PT with different time points of induction and mouse type. Normal brain tissue was stained in red, white the infarct lesion remained unstained. We investigated the infarct volume in TTC stained coronal brain sections after PT. Although the central parts of infarction in brain tissue were lost due to the approach of floating-section staining, the transition from normal tissue to infarction was clearly observed in TTC staining.

To confirm whether Ninj1 contributes to brain damage, we examined the infarct size between WT and Ninj1 KO as well as in early phase and late phase. It revealed no significant differences between both type of mouse at day 3, although a slightly larger infarct size is shown in Ninj1 KO mouse at day 14.

Therefore, these data showed that PT infarct areas, corresponding to WT mouse as well as the Ninj1 KO mouse were not affected in both mouse type in early phase, even though it was more severe in Ninj1 KO mouse than WT in late phase the recovery stage.
As the graph shown, the ration of the infarct size in late phase after photothrombotic injury increased 2.3 fold in Ninj1 KO mice.

Taken together, it comes to a conclusion that TTC staining results implied Ninj1 might be associated with recovery of damaged brain after

focal ischemic stroke.

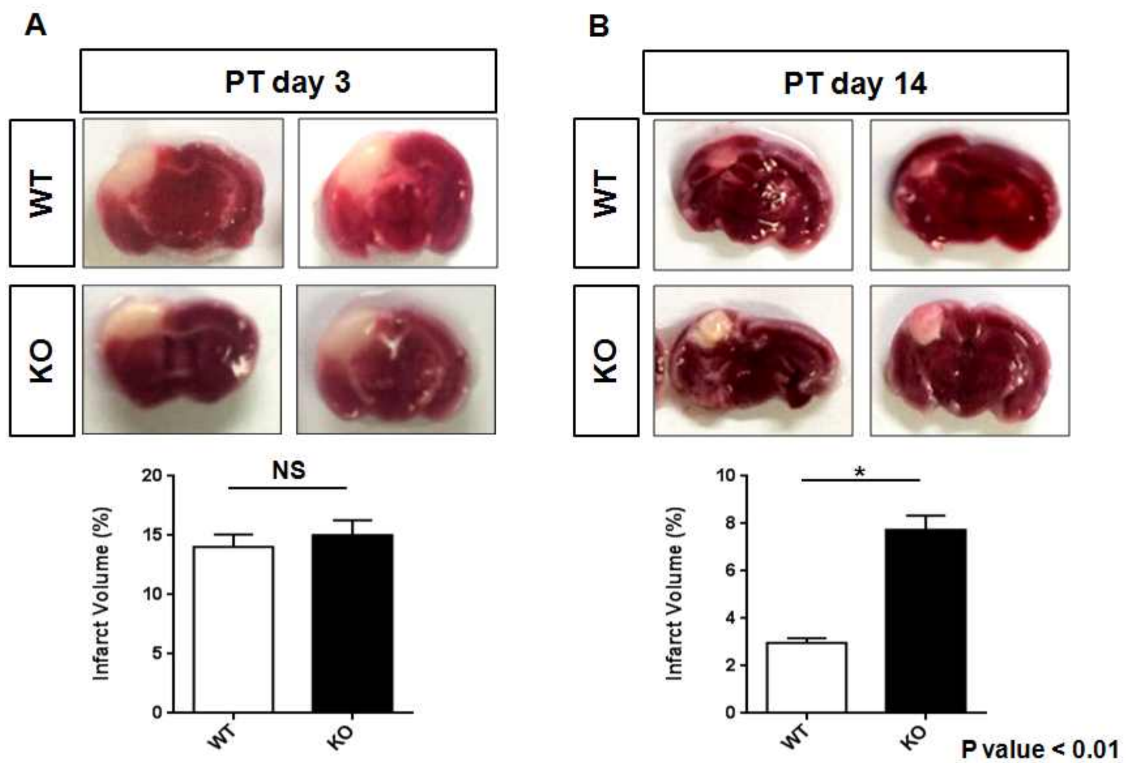


Figure 13. Ninjurin1 KO mice showed larger infarcted tissue compared to WT in the late phase of photothrombosis

(A, B) Representative images of TTC stained ischemic brains from mouse killed at 3 and 14 days after ischemic stroke. The infarction in Ninj1 KO were significantly larger than that of WT mice.

Ⅳ. DISCUSSION

Under the ischemic stroke condition, inflammation appears to contribute to cerebral ischemic injury. It can be caused due to focal cerebral ischemia, followed by excitotoxicity, oxidative stress, BBB dysfunction, microvascular injury, hemostatic activation, post-ischemic inflammation and finally cell death of neurons, glial and endothelial cells. (Zoppo and Hallenbeck et al., 2000). Although the mechanisms underlying BBB disruption under pathological conditions were recently rated as high priority for stroke research, (Meairs S et al., 2006) related pathophysiological processes associated with cerebral damage is still fully understood.

In previous research showed endothelial damage as a potential mechanism for BBB breakdown in a thromboembolic model of focal cerebral ischemia (Krueger M et al., 2013). In addition, tight junctions are well-developed between adjacent endothelial cells of blood vessels in the CNS, and play a central role in establishing the BBB. In here, Claudin-5 is a major cell adhesion molecule of tight junctions in brain endothelial cells.

These cellular events collaboratively contribute to ischemic brain injury.

From this view point, our findings suggest the possibility that the

photothrombotic cerebral injury mediated by Ninj1 through BBB protection.

With the use of PT mice model and mice lacking Ninj1 gene, the present study provides causal evidence that, during the focal ischemic stroke, Ninj1 is largely responsible for the early loss of BBB integrity. Our studies also support a proinflammatory role for Ninj1 in promoting cerebrovascular leukocyte-endothelial adherence and macrophage transmigration into the brain penumbra area. Collectively, these effects indicate that Ninj1 released from intravascular macrophage and Claudin 5 as a tight junction in endothelial cell contributes significantly to the final lesion size after photothrombotic cerebral injury.

. In order to identify the role of Ninj1 in the ischemic stroke, we sampled mice brains in early phase after focal brain injury induced by PT, and examed the Ninj1 level during ischemia stroke.

We first check the Ninj1 expression by RT-PCR and western blot analysis in brain lysate from the PT induction as a model of ischemic stroke. Ninj1 was expressed in infarct lesion of PT brain and was up-regulated in ischemic stroke condition.

With immunohistochemistry experiments, we proved the Ninj1 was weakly expressed in endothelial cells in the PT lesion core. However, many myeloid cell especially macrophage co-localized with Ninj1 were increased in the penumbra area in response to photothrombotic ischemia.

Unique to our study was the finding that the PT mice lacking Ninj1 exhibited a increase in Evans blue permeability that was significant relative to their BBB disruption. Moreover, Claudin 5 was completely detectable from the WT brain but not in the Ninj1 KO brain endothelial cells. These results strongly implicate that Ninj1 as a contributor to protect BBB intensity during ischemic stroke.

In the final analysis, we performed the TTC staining. The PT mice exhibited smaller infarcts size and lower post-ischemic neurological deficit in WT compared to Ninj1 KO. These data suggest that Ninj1 is an important molecule that mediates brain damage in ischemic brain.

These findings would suggest that, under the condition of ischemic focal stroke wherein macrophage could access the ischemic zone, the contribution of Ninj1 to vascular and parenchymal injury may be up-regulated. Indeed, after PT in mice, Ninj1 expression was localized to the penumbra with macrophage and was weakly with endothelial cells in the infarct core. In addition, experiments in WT and Ninj1 KO mice after photothrombotic injury, showed that Ninjurin1 leads to the BBB protection.

Our data demonstrate that Ninj1 reduces BBB leakage and decreases lesion volume in ischemic brain by modulating the infiltrating of macrophage through the tight junction Claudin 5. Therefore, administration of Ninj1 may provide a therapeutic avenue for treatment of

disruption of BBB permeability in the ischemic stroke.

V. REFERENCES

Araki, Toshiyuki, and Jeffrey Milbrandt. "Ninjurin, a novel adhesion molecule, is induced by nerve injury and promotes axonal growth." Neuron 17.2 (1996): 353-361.

Araki, Toshiyuki, et al. "Mechanism of homophilic binding mediated by ninjurin, a novel widely expressed adhesion molecule." Journal of Biological Chemistry 272.34 (1997): 21373-21380.

Ahn, Bum Ju, et al. "Ninjurin1 deficiency attenuates susceptibility of experimental autoimmune encephalomyelitis in mice." Journal of Biological Chemistry 289.6 (2014): 3328-3338.

Ahn, Bum Ju, et al. "Ninjurin1 enhances the basal motility and transendothelial migration of immune cells by inducing protrusive membrane dynamics." Journal of Biological Chemistry 289.32 (2014): 21926-21936.

Manaenko, Anatol, et al. "Comparison Evans Blue injection routes: intravenous versus intraperitoneal, for measurement of blood–brain barrier in a mice hemorrhage model." Journal of neuroscience methods 195.2 (2011): 206-210.

Gu, Yong, et al. "Caveolin 1 regulates nitric oxide mediated matrix metalloproteinases activity and blood–brain barrier permeability in focal cerebral ischemia and reperfusion injury." Journal of neurochemistry 120.1 (2012)

Kim, Kwang Sik. "Mechanisms of microbial traversal of the blood‐brain barrier." Nature Reviews Microbiology 6.8 (2008): 625-634.

Lakhan, Shaheen E., Annette Kirchgessner, and Magdalena Hofer. "Inflammatory mechanisms in ischemic stroke: therapeutic approaches." J Transl Med 7.1 (2009): 97.

Danton, Gary H., and W. Dalton Dietrich. "Inflammatory mechanisms after ischemia and stroke." Journal of Neuropathology & Experimental Neurology 62.2 (2003): 127-136.

Jin, Rong, Guojun Yang, and Guohong Li. "Inflammatory mechanisms in ischemic stroke: role of inflammatory cells." Journal of leukocyte biology 87.5 (2010): 779-789.

Sandoval, Karin E., and Ken A. Witt. "Blood-brain barrier tight junction permeability and ischemic stroke." Neurobiology of disease 32.2 (2008): 200-219.

Li, Fuhai, et al. "Delayed triphenyltetrazolium chloride staining remains useful for evaluating cerebral infarct volume in a rat stroke model." Journal of Cerebral Blood Flow & Metabolism 17.10 (1997): 1132-1135.

Nidavani, Ramesh B., A. M. Mahalakshmi, and Mallappa Shalawadi. "Vascular permeability and Evans blue dye: a physiological and pharmacological approach." Journal of Applied Pharmaceutical Science Vol 4.11 (2014): 106-113.

Durukan, Aysan, and Turgut Tatlisumak. "Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia." Pharmacology Biochemistry and Behavior 87.1 (2007): 179-197.

Rosell, Anna, et al. "MMP-9–Positive Neutrophil Infiltration Is Associated to Blood–Brain Barrier Breakdown and Basal Lamina Type IV Collagen Degradation During Hemorrhagic Transformation After Human Ischemic Stroke." Stroke 39.4 (2008): 1121-1126.

Sandoval, Karin E., and Ken A. Witt. "Blood-brain barrier tight junction permeability and ischemic stroke." Neurobiology of disease 32.2 (2008): 200-219.

Wang, Tiannan, et al. "Controlling the volume of the focal cerebral ischemic lesion through photothrombosis." Am J Biomed Sci 2 (2010): 33-42.

Li, Hailong, et al. "Histological, cellular and behavioral assessments of stroke outcomes after photothrombosis-induced ischemia in adult mice." BMC neuroscience 15.1 (2014): 58.

Zhang, Z. G., et al. "Angiopoietin-1 reduces cerebral blood vessel leakage and ischemic lesion volume after focal cerebral embolic ischemia in mice." Neuroscience 113.3 (2002): 683-687.

Aoki, Toshiaki, et al. "Blood-brain barrier disruption and matrix metalloproteinase-9 expression during reperfusion injury mechanical versus embolic focal ischemia in spontaneously hypertensive rats." Stroke 33.11 (2002).

Jiao, Haixia, et al. "Specific role of tight junction proteins claudin-5, occludin, and ZO-1 of the blood‐brain barrier in a focal cerebral ischemic insult." Journal of Molecular Neuroscience 44.2 (2011): 130-139.

Kramer, Martin, et al. "TTC staining of damaged brain areas after MCA occlusion in the rat does not constrict quantitative gene and protein analyses." Journal of neuroscience methods 187.1 (2010): 84-89.

Nitta, Takehiro, et al. "Size-selective loosening of the blood-brain barrier in claudin-5‐deficient mice." The Journal of cell biology 161.3 (2003): 653-660.

Hou, Sheng Tao, et al. "Semaphorin3A elevates vascular permeability and contributes to cerebral ischemia-induced brain damage." Scientific reports 5 (2015).

Heuschmann, Peter U., et al. "Frequency of thrombolytic therapy in patients with acute ischemic stroke and the risk of in-hospital mortality the German Stroke Registers Study Group." Stroke 34.5 (2003): 1106-1112.

Cha, Jong-Ho, et al. "AKAP12 mediates barrier functions of fibrotic scars during CNS repair." PloS one 9.4 (2014): e94695.

Krueger, Martin, et al. "Blood‐brain barrier breakdown involves four distinct stages of vascular damage in various models of experimental focal cerebral ischemia." Journal of Cerebral Blood Flow & Metabolism 35.2 (2015): 292-303.

국 문 초 록

Nerve injury induced protein 1 (Ninjurin1, Ninj1) 은 친동종 결합 (homophilic adhesion)을 하는 막단백질로써 말초신경계에서의 세포간 결합을 통해 신경돌기의 연장을 유도한다. 게다가 Ninj1은 다발성경화증 (Multiple sclerosis, MS)의 동물 모델인 experimental autoimmune encephalomyelitis (EAE) 상황에서 발현이 증가되어 있으며, 백혈구의 혈관외이행 (leukocyte trafficking)을 매개하는 것으로 알려져 있다. 이 전까지는 주로 EAE 발병에서의 Ninj1 기능에 대한 연구가 주로 진행되었으며 중추신경계에 관여하는 Ninj1의 역할에 대한 연구는 미미한 상황이다. 이러한 연구를 바탕으로 허혈성 뇌졸중과 같은 중추신경계 염증 질환에서의 Ninj1 의 기능을 규명하고자 하였다.

뇌졸중 모델을 구축하기 위해 8-10주령의 C57BL/6 마우스에 Photothrombosis(PT) 유발을 수행하였다. 면역형광염색을 통해 PT 유발 마우스의 뇌 조직과 대조군의 뇌 조직에서의 Ninj1 발현 위치를 확인하였다. 흥미롭게도 대조군의 뇌 조직에서는 뇌척수막의 대식세포에서 Ninj1의 발현을 확인하였고 PT 유발 모델의 뇌 조직에서는 PT 유발 초기 단계에서 손상된 조직 주변으로 반음영 부위(penumbra area)에서 주로 발현이 되는 것과 손상된 조직의 내피세포에서 약하게 발현하는 것을 확인하였다. 뇌졸중 진행과정에서 말기 단계로 갈수록 손상된 주변 조직으로 Ninj1을 발현하는 면역세포의 수가 증가하였다.

Ninj1을 발현하는 세포 타입을 확인하기위해 CD45(백혈구), F4/80

(대식세포)와 같은 면역세포 표지인자와 Ninj1을 이중 면역형광염색을 수행하였다. 그 결과 PT유발 후 초기 단계에서 골수성 면역세포인 백혈구, 특히 대식세포에서 Ninj1의 발현을 확인할 수 있었다. 게다가, Ninj1 KO 마우스에서의 국소적인 뇌 손상 부위에서는 Claudin 5에 의한 뇌혈관장벽의 붕괴와 그로인한 회복 단계에서의 경색부의 크기 증가를 관찰할 수 있었다.

이상의 결과를 통해 Ninj1이 뇌졸중 질환 조직에서의 대식세포의 분포를 조절하고 뇌 내피세포에 존재하는 밀착연접 단백질의 발현을 조절하여 뇌혈관 장벽의 보호에 관여하는 것으로 생각된다. 따라서 본 연구를 통해 Ninj1이 뇌졸중의 발병 정도를 조절할 경우 허혈성 뇌졸중과 같은 치료제 발굴을 위한 주요 후보 단백질이 될 수 있음을 제안하였다.

주요어 : Ninj1, Photothrombosis, Ischemic stroke, macrophage, Blood-Brain Barrier, CNS injury

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